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By: William Ramsey

Date: 3/15/03

Atty Docket No.: D/2000.616 US C2

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

CONN ET AL.

Serial No.: 09/998,619 Group Art Unit: 1653

Filed: November 30, 2001 Examiner: Liu, S.

For: PURIFICATION OF HUMAN TROPONIN I

Assistant Commissioner of Patents
Washington, D.C. 20231

Dear Sir:

**DECLARATION OF PHILIP ROPP PURSUANT TO 37 CFR §1.132**

1. I, Philip Ropp, residing in North Carolina, state that the following is true and correct to the best of my knowledge, under penalty of perjury. These statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 USC and that such willful false statement may jeopardize the validity of the instant patent application or any patent issuing thereon.

2. I am an Associate Director of Purification Development for Diosynth RTP, Inc. Diosynth RTP is a company of Akzo Nobel NV, the assignee of record. My office is located in Cary, North Carolina, USA.

3. My educational background includes a doctorate in biochemistry from University of North Carolina at Chapel Hill.

4. As an Associate Director for Purification Development, I am involved in application

**B. REMARKS**

The Advisory Action issued by the Examiner on September 24, 2003 stated that the Amendment of August 15, 2003 was not entered. Applicants have now amended Claim 1 and made argument to overcome the rejections. Claims 1, 3-9, and 13-20 are pending.

**1. 35 USC §112, 2nd ¶**

Claims 1-9 and 13-20 stand rejected under 35 USC §112, 2nd ¶, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

Applicants have amended the claims and submitted the Declaration of Phil Ropp (attached as Exhibit A) in support of Applicants assertion that the claims are clear as written.

The amended claims language is "[a] method of preparing naturally occurring Troponin I for purification, which method comprises protecting free sulfhydryl groups of Troponin I under reducing conditions, wherein the free sulfhydryl groups are protected by sulfitolyzation." The added phraseology of "for purification" was inherent from the disclosure and does not act to further limit the claims. However, the phraseology does point out that we are preparing the naturally occurring TnI for purification, thereby overcoming the rejections.

**2. Double Patenting and Provisional-Type Double Patenting**

of the technology of the instant application.

5. It is my opinion that people ordinarily skilled in the art understand the meaning of "protecting" and "deprotecting" sulfhydryl groups. These terms are also commonly referred to as "blocking" and "deblocking" (Chan 1968, Kim et al. 1997, Mukhopadhyay 2000).

6. It is well known in the art that the purification of proteins that contain multiple cysteine residues can be complicated due to the formation of disulfide linked aggregates and/or disulfide isomers. To simplify the purification process the disulfide bonds are routinely reduced with a reducing agent. This allows for the purification of a homogeneous form of the protein. During the purification the protein needs to be maintained in a reducing environment to prevent the spontaneous reformation of disulfied bonds or the free sulfhydryl groups can be protected by reacting the reduced protein with chemical agents that react with the sulfhydryl group.

7. Part of the novelty of the instant invention was in protecting the free sulfhydryl groups. By protecting the free sulfhydral groups, the protein no longer needs to be maintained in a reducing environment.

8. Further novelty is found in that the sulfhydryls are reversibly protected. In this manner, the protecting group can be removed at some point in the process.

9. Oxidative sulfitolysis is a cyclic process in which disulfide bonds are reduced by a reducing agent, in an embodiment of the patent, the reducing agent is sulfite, to generate one S-sulfonated cysteine residue and one half-cystine residue. The oxidizing agent, in and embodiment of this patent, is tetrathionate. The half cysine residue is converted back into a disulfide starting the cycle again. In the presence of excess reagents, this process continues until all cysteins have been converted into the S-sulfonated form (see figure 1A of the patent).

10. It is well known in the art that protected cysteine S-sulfonates are produced by oxidative sulfitolysis. They, themselves disulfides. These cysteine S-sulfonates can then be readily reduced, or deprotected, back to cysteine, with a free sulfhydral, by any common reducing reagents such as dithiothreitol, 2-mercaptoethanol, cysteine, or gutathione to name a few. In this patent, the deprotection, or reduction, of the sulfonated protein is effected by dithiothreitol (see figure 11 of the patent).

11. I have enclosed copies of three prior art articles that disclose protecting/deprotecting and/or blocking/deblocking. (Referenced below) Accordingly, it is my opinion that one skilled in the art would find the claims definite and understand the step(s) for protection/deprotection.

**A method for the complete S sulfonation of cysteine residues in proteins,** *Biochemistry Volume 7, Issue 12, December 1968, Pages 4247-4253*
Chan, W W-C

**Molecular assembly of the extracellular domain of P2X2, an ATP-gated ion channel,** *Biochemical And Biophysical Research Communications, Volume 240, Issue 3, November 26, 1997, Pages 618-622*
Kim, M; Yoo, O J; Choe, S

**Reversible protection of disulfide bonds followed by oxidative folding render recombinant hCGbeta highly immunogenic,** *Vaccine, Volume 18, Issue 17, March 6, 2000, Pages 1802-1810*
Mukhopadhyay, A

08-26-03
Date

Signature

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 240, 618–622 (1997)
ARTICLE NO. RC977713

# Molecular Assembly of the Extracellular Domain of $P2X_2$, an ATP-Gated Ion Channel

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Received September 29, 1997

**We have produced the putative extracellular domain (ECD) of the ATP-gated ion channel, $P2X_2$, in a bacterial expression system. The hexahistidine-tagged protein was purified by immobilized metal affinity chromatography and refolded by sulfitolysis and dialysis. We demonstrate that $P2X_2$-ECD forms a stable tetramer in solution by gel filtration chromatography, dynamic light scattering and analytical sedimentation centrifugation. [$\alpha$-$^{32}$P]ATP has been covalently cross-linked by UV irradiation to the $P2X_2$-ECD and this binding is specific and competable by antagonists suramin and cibacron blue. These results indicate that the binding affinity among $P2X_2$-ECD subunits is appreciably stronger than 3.4 $\mu$M (0.1 mg/ml), implying that the extracellular domain of $P2X_2$ is primarly responsible for tetramerization of whole $P2X_2$ and thus probably plays a role in determining homo- and heteromerization specificity of P2X channel subunits.** © 1997 Academic Press

P2 receptors are cell surface proteins which mediate the physiological effects of extracellular ATP in mammalian cardiovascular, immune and nervous systems. P2 receptor subtypes have been defined according to the relative potency of various nucleotide agonists and by differences in signal transduction mechanisms. By these criteria, five main subtypes have been proposed to exist: P2Y, P2U, P2T, P2X and P2Z (1-3). Physiological studies and molecular cloning of functional cDNAs encoding many of these receptors have revealed a large family of G-protein coupled receptors (P2Y, P2U, and possibly P2T) and ion channels (P2X and P2Z) that are responsive to ATP. At present, there are 7 known subtypes of P2X receptors sharing approximately 40% sequence identity ($P2X_1$ - $P2X_7$) (4-14). The P2X receptors (including the recently identified $P2X_7$, formerly P2Z) contain an intrinsic ion channel which is approximately equally permeable to small cations ($Na^+$, $K^+$ and $Ca^{2+}$) and is directly gated by ATP. Expression of a single cDNA clone in *Xenopus* oocytes or transfected mammalian cells is sufficient to direct the synthesis of functional, ATP-gated ion channels on the surfaces of these cells. In these expression systems there are phenotypic differences among the receptors, particularly with respect to rates of desensitization and relative sensitivities to the agonist $\alpha\beta$-meATP and the antagonists PPADS and suramin (11, 15).

The largest family of ligand-gated ion channels includes the acetylcholine, serotonin, glycine, and $GABA_A$ receptors. Based largely on studies of the acetylcholine receptor, these proteins are believed to consist of 5 subunits arranged in a toroidal fashion to delineate a central aqueous pore. Each subunit consists of a large amino-terminal extracellular ligand-binding domain followed by four transmembrane segments. The P2X receptors define a novel structural motif for ligand-gated ion channels in which each subunit is proposed to have two transmembrane domains flanking a large putative extracellular loop. The occurrence of only two transmembrane domains is reminiscent of the inwardly-rectifying potassium channels, *mec* or mechanosensory channels from *C. elegans*, and amiloride-sensitive sodium channels which are believed to be composed of four subunits (16-19).

$P2X_2$ has been recently characterized as one of the P2X receptor family members, originally isolated from a cDNA library of rat pheochromocytoma PC12 cells differentiated with nerve growth factor (5). $P2X_2$ receptors, which has shown expression in several tissues, bladder, brain, spinal cord, intestine and vas deferens, are sensitive to the antagonist suramin. $P2X_2$ functions as cation-selective ATP-gated ion channel, which is thought to be activated by ATP binding to the extracellular domain of $P2X_2$ (5).

Little is known about the family of $P2X_2$ receptors on the molecular level. Which regions of the receptor form the ligand-binding pocket and how do they inter-

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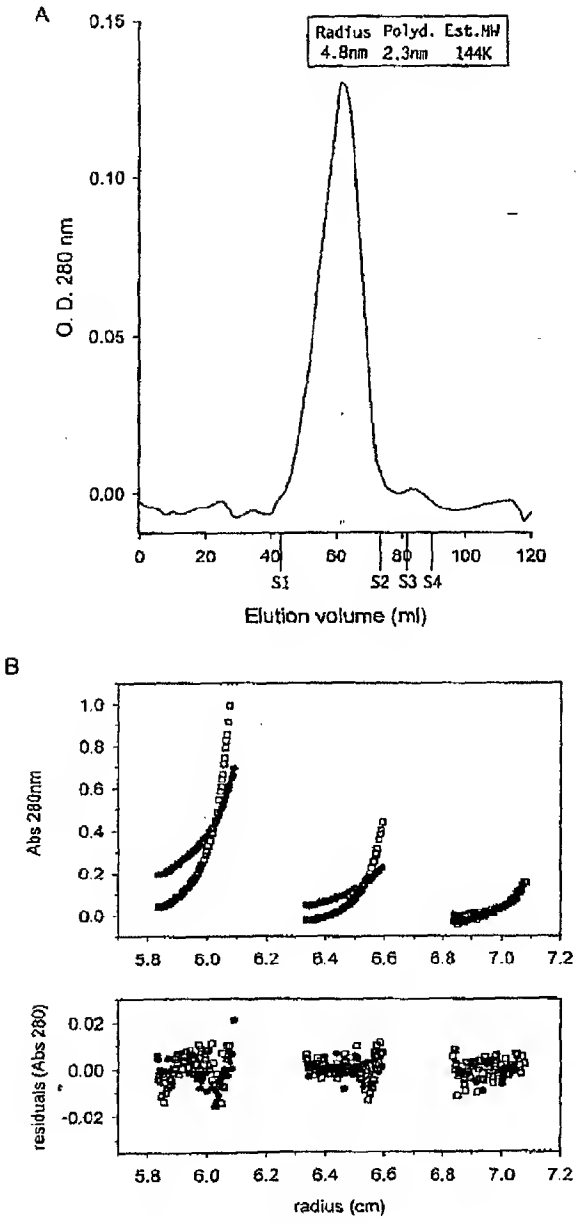


FIG. 4. (A) Chromatogram from gel filtration with FPLC S200 sizing column. The elution volumes of four different molecular weight standards are : S1, Blue dextran (2,000 K, 43.58 ml); S2, Bovine serum albumin (67 K, 73.27 ml); S3, Ovalbumin (43 K, 80.15 ml); S4, Chymotrypsin (25 K, 89.78 ml). (inset) data from dynamic light scattering measurement (Biotage) (B) Equilibrium sedimentation profiles of $P2X_2$-ECD at 7,000 and 10,000 rpm and at 3 different protein concentrations (0.1, 0.2, and 0.5 mg/ml) at the start of centrifugation were shown in absorbance at 280 nm. The bottom panel is the residuals after curve fitting, which shows no systematic errors.

topological similarity of P2X receptor channels to inward rectifying ion channels, the tetrameric form of $P2X_2$-ECD may represent the naturally assembled form of the $P2X_2$ receptor channel. It also implies that the extracellular ligand-binding domain of $P2X_2$ receptors may play a major role in determining subunit specificity and tetramer formation *in vivo*.

$P2X_2$-ECD contains ten cysteines, which may form up to five intramolecular disulfide bonds. Blocking the formation of disulfide bonds by sulfitolysis during protein refolding is essential to yield soluble and correctly folded protein. Based on the functional and structural parameters we have tested, our $P2X_2$-ECD behaves like the natural $P2X_2$ channel in the membrane indicating that it has achieved a native-like fold. N-terminal amino acid sequencing revealed that after the hexahistidine tag was removed by thrombin digestion, and the new N-terminal residue was $Ser^{54}$. In addition, mild trypsin treatment eliminated 25 to 27 N-terminal residues, leaving $Val^{79}$ and $Gly^{81}$ at the N-terminus of the truncated protein (not shown). These sites are apparently trypsin-sensitive and solvent-exposed, possibly bridging the N-terminal segment ($Ser^{54}$ to $Val^{79}$) to the rest of the polypeptide chain. Interestingly, this segment appears to be important in protein refolding because the truncated protein does not refold in the same manner.

We have observed specific binding of ATP to the purified $P2X_2$-ECD in photoaffinity crosslinking experiments. Although these crosslinking experiments cannot be used for quantitative determinations, due to the possible decrease in photocrosslinking efficiency in different assay conditions, it is clear that an excess amount of nonradioactive ATP prevents the crosslinking of radioactive ATP. For instance, denatured $P2X_2$-ECD shows no difference in radioactivity between crosslinking in the presence or absence of excess amount of nonradioactive ATP, indicating that the nature of nonspecific ATP binding is irrelevent to occupancy of specific ATP-binding site. The decrease of ATP-specific labeling by suramin and cibacron blue is also consistent with the interpretation that the access to the ATP-binding site is inhibited by these antagonists. Direct measurement of binding affinity of $P2X_2$-ECD to various agonists and antagonists is currently underway. Preliminary data suggest that the presence of divalent cations might be crucial for measurable binding of the ligands.

The amino acid(s) crosslinked to ATP are likely to be similar among those of the ATP-binding pockets of related receptors, and they probably play a structurally similar, crucial role for the channel activation of P2X receptor. With the soluble preparation of functional $P2X_2$-ECD, it will be possible to identify those positions biochemically after photoaffinity labeling. Furthermore, those positions can be mutated individually and single-point mutants can be characterized for its ATP-

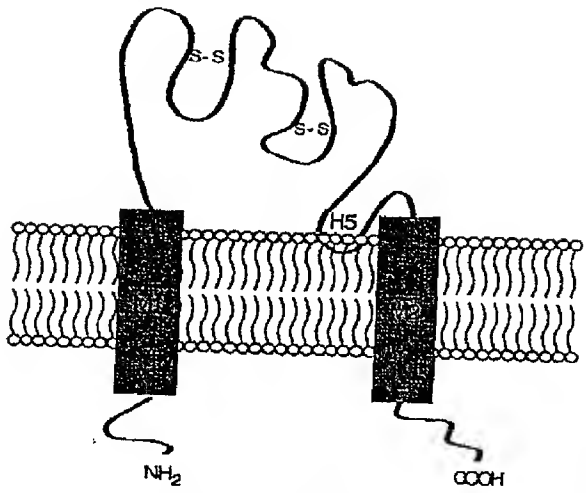


FIG. 1. Predicted topology of the $P2X_2$ receptor (5). The $P2X_2$ receptor is shown with both N and C termini in the cytoplasm. Two putative membrane-spanning segments (M1 and M2) traverse the lipid bilayer of the plasma membrane and are connected by a hydrophilic segment of ~270 amino acids that encompasses the ligand-binding domain ($P2X_2$-ECD).

act with nucleotide on the molecular level? How do the P2X receptor subunits interact with each other to form homomeric or heteromeric complexes? In the present study, we have sought to address some of these questions through the characterization of the ligand-binding properties and quaternary structure of the extracellular domain of $P2X_2$ ($P2X_2$-ECD).[2] This protein fragment has been overexpressed in bacteria, solubilized in urea, and refolded. Analytical ultracentrifugation, dynamic light scattering, and gel filtration chromatography indicate that the $P2X_2$-ECD forms a stable tetramer in solution. We demonstrate this purified, refolded $P2X_2$-ECD now provides a direct means to biochemically and structurally characterize the ATP binding site of the $P2X_2$ receptor.

## MATERIALS AND METHODS

*Construction of $P2X_2$-ECD plasmid.* The $pP2X_2$-ECD encodes a sequence of extracellular ATP binding domain of $P2X_2$ except H5, under the regulation of T7 polymerase of a bacterial overexpression vector pET28a (Novagen). Fig. 1 shows the putative topological model of $P2X_2$ (5). A fragment of the open reading frame of the $P2X_2$-ECD ($Lys^{53}$ to $Lys^{308}$) was amplified by PCR using *pfu* polymerase and the primers 5′-TG CCG CGC GGC AGC CAT ATG AAA AGC TAC CAG GAC AGC -3′ and 5′-CG ACG GAG CTC GAA TTC GGA TCC TTA TTT GAT GAG AGT TCG ACT-3′ and using $P2X_2$ cDNA (5) as a template (NdeI and BamHI restriction sites are underlined). The PCR product was subcloned into the vector pET28a which fuses the $P2X_2$-ECD in frame to an amino-terminal methionine, hexahistidine tag, and thrombin cleavage site, respectively. This cloning scheme results in the addition of a stop codon after the codon for $Lys^{308}$.

*Expression of $P2X_2$-ECD.* The plasmid ($pP2X_2$-ECD) was transformed into *E.coli* BL21 (DE3) for protein expression. Transformants were grown at 37°C in LB medium plus kanamycin (50 μg/ml). The overnight culture was diluted 1:200 in the same medium and grown until $A_{600}$ was 0.6-1.0. Protein expression was then induced at 30°C by the addition of 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of induction, cells were pelleted and stored at −70°C.

*Purification of $P2X_2$-ECD.* Cells were lysed by a combination of lysozyme treatment and sonication. Cell pellets were thawed quickly at 37°C, and resuspended in lysis buffer (20 mM Tris, pH 7.0, 150 mM NaCl, 2% Triton X-100) of 10 times the wet volume. Lysozyme (100 μg/ml final) was added to the cell suspension and the mixture was incubated for 30 min on ice. The lysate was sonicated, and cell debris was removed by centrifugation at 10,000 × g for 20 min. The pellet containing $P2X_2$-ECD was washed twice in wash buffer (20 mM Tris, pH 7.0, 150 mM NaCl, 2 M urea, 5 mM β-mercaptoethanol). The pellet was then resuspended in solubilization buffer (20 mM Tris, pH 8.0, 8.0 M urea, 150 mM NaCl, 5 mM β-mercaptoethanol) and by rocking gently at room temperature for 1 h. Insoluble material was removed by centrifugation at 10,000 × g for 40 min at 4°C, and the soluble fraction was subsequently subjected to Ni-affinity chromatography. The soluble fraction was incubated for 2 h with Ni-NTA resin which was pre-equilibrated in solubilization buffer. Unbound protein was removed by washing the resin with 20 mM Tris, pH 8.0, 8.0 M urea, 150 mM NaCl, 5 mM imidazole. Bound protein was eluted with the same buffer containing 250 mM Imidazole. The concentration and purity of the eluted fractions were estimated by Bradford method (20) and by a 15% SDS-PAGE.

*Sulfitolysis and refolding of $P2X_2$-ECD.* Prior to sulfitolysis, the protein solution was adjusted to pH 9.0 by adding 0.5 N NaOH, and incubated with sodium sulfite (200-fold molar excess) for 10 min. Then NTSB stock solution (21) was added in the amount of 0.5 ml $\mu mole^{-1}$ protein and stirred for 30 min. At 5 min intervals, aliquots (10 μl) were removed and diluted with 0.1 M Tris buffer (pH 9.0). The absorbance at 412 nm was measured to determine the endpoint of the reaction. Urea was removed gradually by dialysis against a minimum of 20 volumes of buffer A (20 mM Tris, pH 9.0, 150 mM NaCl) in steps of 2, 1, 0.5, and 0 M urea. Sulfite groups were removed from $P2X_2$-ECD by dialysis against buffer B (20mM Tris, pH 8.0, 150 mM NaCl) plus 5 mM β-mercaptoethanol. To remove the hexahistidine tag from the amino-terminus of $P2X_2$-ECD, thrombin (1:1000 thrombin:protein by weight) was added to the protein solution and the mixture was dialyzed against buffer B plus 5 mM β-mercaptoethanol and 2.5 mM $CaCl_2$ at 4°C, overnight. Finally, $P2X_2$-ECD protein was purified by gel filtration chromatography (Superdex S200, Pharmacia) in buffer B. Protein identity was confirmed by amino-terminal amino acid sequencing.

*Photoaffinity labeling.* Purified $P2X_2$-ECD (10 μg) was incubated with 2 nM [α-$^{32}$P]ATP at 4°C for 2 h in buffer B plus 2 mM $MgCl_2$. As a control, $P2X_2$-ECD was coincubated with 100 μM nonradiolabeled ATP under the same condition prior to the exposure to the UV irradiation. After incubation, samples were irradiated by a hand-held UV (254 nm) lamp for 10 min at a distance of 5 cm. Protein was precipitated by the addition of 2 volumes of cold acetone and incubation at 4°C, overnight and were subsequently analyzed by SDS-PAGE. Dried gels were autoradiographed at −80°C using X-Omat X-ray film (Kodak).

*Analytical ultracentrifugation.* Sedimentation equilibria were determined with a Optima XL-1 ultracentrifuge (Beckman). Three

[2] Abbreviations: $P2X_2$-ECD, extracellular domain of $P2X_2$; FPLC, fast process liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NTSB, disodium 2-nitro-5-thiosulfobenzoate; IPTG, isopropyl-β-D-thiogalactopyranoside.

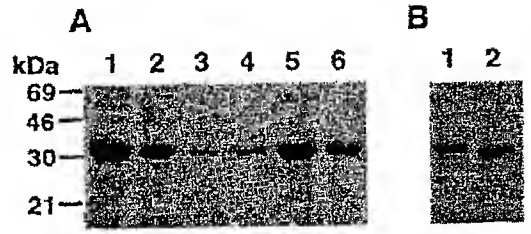


FIG. 2. Purification of $P2X_2$-ECD on Ni-NTA chromatography. Induced proteins were extracted with 8 M urea and the soluble fraction was loaded onto a Ni-NTA column and chromatographed as described in the Materials and Methods. (A) Lane 1, total 8 M urea-solubilized proteins; lane 2, flow-through; lane 3, proteins eluted by low concentration of imidazole; lanes 4-6, eluted fractions with 250 mM imidazole. The positions of molecular size markers are shown. (B) Coomassie stained SDS-PAGE of refolded $P2X_2$-ECD before and after the cleavage of hexahistidine tag. Lane 1, before cleavage (31 kDa); lane 2, after cleavage (29 kDa).

concentrations of $P2X_2$-ECD (0.1, 0.2, and 0.5 mg/ml) in 120 µl volume each were centrifuged at two centrifugation speeds (7 K and 10 K rpm) for 16 h to ensure the equilibrium. Absorbance at 280 nm was measured at 1 h time intervals. Approach to equilibrium was monitored by calculating RUS deviation of each data set compared to the last data set using the program MATCH (Jeff Lazy, National Analytical Ultracentrifugation Facility, Storrs, CT). Data were analyzed and plotted by global nonlinear least squares fitting using the program WINNONLIN (22).

## RESULTS

*Overexpression and purification of $P2X_2$-ECD.* The $P2X_2$-ECD was overexpressed in bacteria with an amino-terminal hexahistidine-tag separable by thrombin cleavage site. $P2X_2$-ECD was induced by 200 µM IPTG at 30°C for 3 h at a level of over 100 mg liter$^{-1}$ bacterial culture, and the induced protein appeared in inclusion bodies. A rapid, single step-purification using Ni-affinity chromatography was performed, yielding approximately 50 mg of $P2X_2$-ECD from 1 L cell culture. The eluted $P2X_2$-ECD was in excess of 95 % purity based on Coomassie staining and migrated with the expected mobility of 31 kDa protein (Fig. 2A). Cysteine sulfhydryl groups were blocked by sulfitolysis and the protein was refolded by the gradual removal of urea. Sulfite groups were then removed from the protein by reduction. After the removal of the amino-terminal hexahistidine tag by thrombin digestion, the resulting protein migrated as a single band with increased mobility at the predicted molecular size of 29 kDa (Fig. 2B). Finally, $P2X_2$-ECD was separated from proteolytic fragments and thrombin by gel filtration chromatography. The identity of $P2X_2$-ECD and the actual cleavage site were verified by N-terminal amino acid sequencing. The amino-terminal amino acid of the final protein preparation was $Ser^{54}$, which is internal to the predicted thrombin cleavage site.

*[α-$^{32}$P]-ATP photoaffinity labeling.* To demonstrate that ATP directly binds to the purified $P2X_2$-ECD, we carried out photoaffinity crosslinking of [α-$^{32}$P]ATP to the purified $P2X_2$-ECD. Refolded $P2X_2$-ECD displayed specific binding to [α-$^{32}$P]ATP and this binding was competable by the antagonists suramin (1 µM) and cibacron blue (10 µM). Purified $P2X_2$-ECD denatured by 8M urea (Fig.3) showed the same level of ATP crosslinking whether an excess amount of nonradioactive ATP was present or not, indicating that denatured $P2X_2$-ECD binds a low level of ATP nonspecifically.

*The molecular size of $P2X_2$-ECD.* We used gel filtration chromatography, dynamic light scattering, and analytical ultracentrifugation to determine the approximate molecular size and therefore the quaternary structure of the refolded $P2X_2$-ECD. Fig. 4A shows the gel filtration chromatography profile. The molecular size was estimated to be approximately 160 kDa based on elution times of four molecular weight standards. Dynamic light scattering, which measures the translational diffusion coefficient of the protein, indicated an approximate molecular weight of 144 kDa (Fig. 4A, inset). Equilibrium sedimentation centrifugation was performed because it gives an estimate of molecular size which is independent of the molecular shape of the protein. The direct measurement of protein density by equilibrium centrifugation (Fig. 4B) shows that the majority of the protein is the form of tetramer (132 kDa) with a minor portion of octameric form. The octamer might be a crosslinked artifact of tetrameric form. These results indicate $P2X_2$-ECD forms a stable tetramer in solution at a protein concentration at as low as 0.1 mg/ml (3.4 µM for monomeric form).

## DISCUSSION

We have shown that $P2X_2$-ECD can be refolded to a stable tetrameric form in solution. In light of the

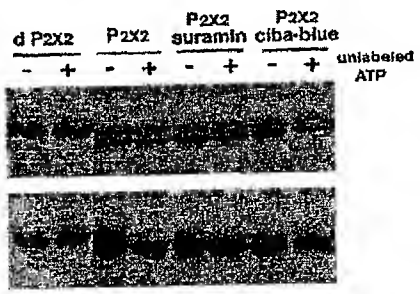


FIG. 3. Photoaffinity labeling of $P2X_2$-ECD with [α-$^{32}$P]ATP. Samples were incubated with 2 nM [α-$^{32}$P]ATP in the presence or absence of competitor, and compared to those incubated in the presence of excess amount of nonradioactive ATP. d $P2X_2$ denotes denatured $P2X_2$-ECD by 8 M urea. Suramin (1 µM) and Cibacron blue (10 µM) have been used. Coomassie stained (upper panel) and autoradiogram (lower panel) are shown.

binding properties and abilities to gate the $P2X_2$ channel. Correlation between these two properties will provide useful information to understand the mechanism underlying the channel regulation by the ligand.

## ACKNOWLEDGMENTS

The authors are grateful to Drs. David Julius and Laura England for the gene construct of $P2X_2$ and insightful discussion, and also for many suggestions in preparing the manuscript. The work was in part supported by The Council for Tobacco Research.

## REFERENCES

1. Abbracchio, M. P., and Burnstock, G. (1994) *Pharmacol. Ther.* **64**, 445–475.
2. Fredholm, B. B. (1994) *Pharmacol. Rev.* **46**, 143–156.
3. Brake, A., and Julius, D. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 519–541.
4. Valera, S., Hussy, N., Evans, R. J., Adami, N., North, A., Suprenant, A. and Buell, G. (1994) *Nature* **371**, 516–518.
5. Brake, A. J., Wagenbach, M. J., and Julius, D. (1994) *Nature* **371**, 519–523.
6. Chen, C.-C., Akopian, A. N., Sivilotti, L., Colquhoun, D., Burnstock, G. and Wood, J. N. (1995) *Nature* **377**, 428–431.
7. Lewis, C., Neidhart, S., Holy, C., North, R. A., Buell, G. and Suprenant, A. (1995) *Nature* **377**, 432–435.
8. Bo, X., Zhang, Y., Nassar, M., Burnstock, G. and Schoepfer, R. (1995) *FEBS Lett.* **375**, 129–133.
9. Buell, G, Lewis, C., Collo, G. and Burnstock, G. (1996) *EMBO J.* **15**, 55–62.
10. Soto, F., Garcia-Guaman, M., Gomez-Hernandez, J. M., Hollmann, M., Karschin, C., and Stuhmer, W. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3684–3688.
11. Collo, G., North, R. A., Kawashima, E., Merlo-Pich, E., Neidhart, S., Suprenant, A., and Buell, G. (1996) *J. Neurosci.* **16**, 2495–2507.
12. Garcia-Guaman, M., Soto, F., Laube, B., and Stuhmer, W. (1996) *FEBS lett.* **388**, 123–127.
13. Soto, F., Garcia-Guaman, M., Karschin, C., and Stuhmer, W. (1996) *Biochem. Biophy. Res. Comm.* **223**, 456–460.
14. Suprenant, A., Rassendren, F., Kawashima, E., and North, R. A., and Buell, G. (1996) *Science* **272**, 735–738.
15. Evans, R. J., Lewis, C., and Buell, G. (1995) *Mol. Pharmacol.* **48**, 178–183.
16. Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993) *Nature* **362**, 127–133.
17. Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V., and Hebert, S. C. (1993) *Nature* **362**, 31–38.
18. Huang, M., and Chakfie, M. (1994) *Nature* **367**, 467–470.
19. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J-D., and Rossier, B. C. (1994) *Nature* **367**, 463–467.
20. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
21. Thannhauser, T. W., Konishi, Y., and Scheraga, H. A. (1984) *Anal. Biochem.* **138**, 181–188.
22. Johnson, M. L., Correia, J. A., Yphantis, D. A., and Halvorson, H. R. (1981) *Biophys. J.* **36**, 575–588.

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References

Bar-Eli, A., and Katchalski, E. (1963), *J. Biol. Chem. 238*, 1690.

Benedetti, E., Kossoy, A., Falxa, M. L., and Goodman, M. (1968), *Biochemistry 7*, 4234 (this issue; paper XXVII).

Blout, E. R., and Karlson, R. H. (1956), *J. Am. Chem. Soc. 78*, 941.

Bradley, D. F., Goodman, M., Felix, A., and Records, R. (1966), *Biopolymers 4*, 607.

Burawoy, A., Salem, A. G., and Thompson, A. R. (1952), *J. Chem. Soc.*, 4793.

Gabor, G., Frei, Y., Gegion, D., Kaganowitch, M., and Fisher, E. (1967), *Israel J. Chem. 5*, 193.

Goodman, M., and Benedetti, E. (1968), *Biochemistry 7*, 4226 (this issue; paper XXVI).

Goodman, M., Davis, G. W., and Benedetti, E. (1968), *Accounts Chem. Res. 1*, 275.

Goodman, M., and Kossoy, A. (1966), *J. Am. Chem. Soc. 88*, 5010.

Goodman, M., and Peggion, E. (1967), *Biochemistry 6*, 1533.

Goodman, M., and Toniolo, C. (1968), *Biopolymers* (in press).

Hadzi, D. (1956), *J. Chem. Soc.*, 2143.

Holzwarth, G., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 218.

Katchalski, E., and Sela, M. (1953), *J. Am. Chem. Soc. 75*, 5284.

Morton, R. A., and Stubb, A. L. (1940), *J. Chem. Soc.*, 1347.

Nurmukhametov, R. N., Shigorin, D. N., Kozlov, Y. I., and Puchkov, V. A. (1961), *Opt. Spectry. USSR*, 606.

Oddo, G., and Puxeddu, E. (1906), *Gazz. Chim. Ital. 36II*, 1.

Ospenson, J. N. (1951), *Acta Chem. Scan. 5*, 491.

Reeves, L. W. (1960), *Can. J. Chem. 38*, 748.

Shigorin, D. N. (1953), *Izv. Akad. Nauk SSSR, Ser. Fiz. 14*, 395.

Shigorin, D. N. (1959), *Spectrochim. Acta 14*, 198.

Tinoco, I., Jr. (1964), *J. Am. Chem. Soc. 86*, 297.

Wettermark, G., Langmuir, M. E., and Anderson, D. G. (1965), *J. Am. Chem. Soc. 87*, 476.

# A Method for the Complete S Sulfonation of Cysteine Residues in Proteins*

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ABSTRACT: A new method is described for the complete sulfonation of protein SH groups under mild conditions. The protein is treated with sodium sulfite and catalytic amounts of cysteine in the presence of oxygen and 8 M urea.

When applied to rabbit muscle aldolase, complete sulfonation was obtained within 1 hr. The reaction was shown to be specific for SH groups from studies of the extent of the reaction and the electrophoretic pattern of the product. S-Sulfonated aldolase was enzymatically inactive but after suitable treatment with β-mercaptoethanol was reconstituted to give the fully active enzyme. The 100% regeneration of enzyme activity suggests that the method might be suitable for studies where subsequent recovery of biological activity is desired. In contrast, the S-sulfonated aldolase prepared by two other methods gave little or no activity after similar treatment. The reaction requires the addition of cysteine which may be replaced by β-mercaptoethylamine but not by β-mercaptoethanol or dithiothreitol. Under the conditions studied complete sulfonation occurs in the pH range 7.0–8.5 but little reaction takes place at pH 9.5 or higher. These findings suggest a role for the protonated amino group of cysteine in the reaction mechanism. Lactate dehydrogenase and pepsinogen were also completely sulfonated by this method. It is therefore suggested that the method may be generally applicable to proteins containing cysteine or cystine residues.

Sulfitolysis has been frequently used for the cleavage of disulfide bonds in proteins (Cole, 1967). If the reaction is allowed to proceed in a dissociating medium (*e.g.*, at high concentrations of urea or guanidine hydrochloride) and in the presence of an oxidizing agent, all the half-cystine residues can be converted into the *S*-sulfonate cysteine derivative. The completely S-sulfonated proteins so obtained are useful in the separation of polypeptides since they are stable in neutral and acidic conditions (Swan, 1957). A distinct advantage of

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$$RSSR \xrightarrow{SO_3^{2-}} RSSO_3^- + RS^-$$
$$\xrightarrow{[O]} RSSO_3^-$$

(RSH = cysteine residues in proteins or other SH compound)

the *S*-sulfonate as a blocking group is its ready removal by excess thiol treatment. In this respect it is similar to disulfides.

The method has not been used to any extent on proteins which contain SH but not disulfide bonds. However, the same reactions are involved, regardless of whether disulfide bonds are present or not because in both cases, oxidation is required for complete sulfonation. For the purpose of modifying SH groups in sequence studies of proteins, the alternative methods of alkylation and performic acid oxidation are undoubtedly superior to sulfonation since the modified groups in these methods are stable to acid hydrolysis. Nevertheless, *S*-sulfonate or mixed disulfides must be used as blocking groups whenever it is desirable to remove them at a later stage.

The present work was undertaken in conjunction with studies on the subunits of rabbit muscle aldolase. It has been shown that aldolase can be reversibly dissociated into subunits (Stellwagen and Schachman, 1962; Deal *et al.*, 1963a). Under certain conditions, the subunits will reassociate to give the native enzyme with essentially complete recovery of activity. However, when conventional methods (Swan, 1957; Katsoyannis *et al.*, 1967) were used to prepare S-sulfonated aldolase and the *S*-sulfonate groups were subsequently removed, only little or no enzyme activity could be recovered by reassociation of the subunits. This suggested that these methods might have caused irreversible physical or chemical change to the protein. An attempt was therefore made to find alternative procedures for sulfonation. This paper describes a simple method which leads to complete S sulfonation under mild conditions. A striking property of the method is that a 100% recovery of aldolase activity is realized when the S-sulfonated derivative of aldolase is treated with excess mercaptoethanol. Although most of the results described above were obtained using aldolase, other evidence presented indicates that the method should be generally applicable to proteins containing cysteine or cystine residues.

*Materials and Methods.* Fructose diphosphate aldolase (4.1.2.13) was prepared from rabbit muscle by the method of Taylor *et al.* (1948) and recrystallized three times. Aldolase activity was assayed spectrophotometrically as described by Racker (1947) and the unit of activity was defined as the amount required to cleave 1 μmole of FDP/min. The molecular weight of aldolase was taken as 160,000 (Kawahara and Tanford, 1966; Sia and Horecker, 1968). Lactate dehydrogenase (1.1.1.27) from rabbit muscle (lot 76153, specific activity 425 units/mg) was supplied by Calbiochem. Pepsinogen was a product of Worthington Biochemical Corp. Urea (A.C.S. grade) was recrystallized from 95% ethanol, washed with ether, and dried in an oven at 40°. Cysteine hydrochloride, EDTA, and sodium sulfite were reagent grade and supplied by the Fisher Scientific Co. Tris base and *N*-ethylmaleimide were obtained from Sigma Chemical Corp. The reagent grade guanidine hydrochloride used was made by J. T. Baker and Co. and was found to be completely soluble in water. $^{35}S$-Labeled sodium sulfite (lot 328–118) was purchased from New England Nuclear Corp. The initial specific activity was 10.5 mCi/mmole. It was diluted by adding carrier until the specific activity was close to 100,000 cpm/μmole as determined below.

*Determination of Protein Concentration.* Protein concentration was assayed either spectrophotometrically from the absorption at 280 mμ in 0.1 N NaOH or by the trichloroacetic acid method of Bücher (1947). The extinction coefficient, $E^{1\%}_{290\,m\mu}$, in 0.1 N NaOH was taken to be 9.1 for aldolase (Baranowski and Niederland, 1949) and 8.85 for lactate dehydrogenase (Pfleiderer and Jeckel, 1957). The trichloroacetic acid method was calibrated against the spectrophotometric method using solutions of aldolase. For S-sulfonated proteins which are insoluble in water the method was modified by adding urea to a 2 M final concentration. The modified method was similarly calibrated.

*Determination of Radioactivity.* Aliquots of solutions were dried on filter paper strips or Millipore filters and placed in scintillation vials. To each vial was added 15 ml of a toluene solution containing 4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis[2-(5-phenyloxazolyl)]-benzene per l. The vials were counted in a Nuclear-Chicago, Model Unilux, scintillation counter. Counting efficiency was approximately 60%.

*Measurement of the Incorporation of $^{35}S$-Labeled Sulfite into Proteins.* Three different procedures were used and all gave identical results. In one method, after the reaction the mixture was dialyzed exhaustively against water. The protein was then dissolved in 0.1 N NaOH and protein concentration was determined by measuring the absorption at 280 mμ. Aliquots of the same solution were dried on filter paper strips for radioactivity counting.

Alternatively, after the reaction the protein was precipitated by adding trichloroacetic acid to a final concentration of 10% and centrifuged. The precipitate was washed by redissolving in 1 ml of 90% formic acid, then diluted to 3 ml, and reprecipitated by adding trichloroacetic acid and centrifuged. The washing procedure was repeated three times. The protein was finally washed with acetone and then with ether, dried, and dissolved in 0.1 N NaOH. Protein and radioactivity were determined as described above.

In the third method, a 0.1-ml aliquot of the reaction mixture was added to 3 ml of 10% trichloroacetic acid solution. After 5 min the trichloroacetic acid solution was filtered through Millipore HAWP 02400 filters (pore size 0.45 μ) and washed thoroughly with 10% trichloroacetic acid solution. The filters were dried and counted for radioactivity. The amount of protein was calculated from the concentration in the original reaction mixture assuming complete precipitation and transfer. The specific activity of the $^{35}S$-labeled sulfite used was determined using both Millipore filters and filter paper strips and the appropriate value was used for each method.

*Regeneration of Free SH Groups and Reassociation of the Subunits of Aldolase.* Lyophilized S-sulfonated aldolase (0.5–1.0 mg/ml) was dissolved in 1 M Tris-HCl solution (pH 7.5) containing 4 M urea. β-Mercaptoethanol was added (final concentration 0.7 M) and the mixture was incubated for 2 hr at 25°. The protein subunits were then allowed to reassociate by diluting the mixture ten times with a 1 M Tris-HCl solution (pH 7.5) containing bovine serum albumin (2.5 mg/ml), EDTA (25 mM) and β-mercaptoethanol (10 mM). The enzyme activity was determined after approximately 15 min.

Disc gel electrophoresis was carried out with a Canalco Model 12 apparatus using the polyacrylamide system of Davis (1964). Recrystallized urea was added to all the solutions used (final concentration 8 M) except the buffer in the reservoirs.

## Results

*Incorporation of $^{35}S$-Labeled Sulfite into Aldolase.* The oxidation of SH groups by molecular oxygen is known to be catalyzed by copper and *o*-phenanthroline (Kobashi and Horecker, 1967). Initially this reaction was used to promote the formation of disulfide bonds. Cysteine was added to facilitate the formation of mixed disulfides since for steric reasons not all of the SH groups in aldolase could be expected to form disulfide bonds with one another. In this experiment, the reaction mixture contained $6.3 \times 10^{-3}$ M aldolase, 0.1 M $Na_2{}^{35}SO_3$ (specific radioactivity 112,000 cpm/μmole), $2 \times 10^{-3}$ M $CuSO_4$, 0.1 M Tris-HCl (pH 8.4), and 8 M urea. After incubation at 25° in an open vessel, the mixture was exhaustively dialyzed against water. The specific radioactivity of the dialyzed protein was found to be 19,300 cpm/mg which represents an incorporation of 27.5 sulfonate groups/mole of aldolase. Since aldolase has been shown (Swenson and Boyer, 1957) to contain approximately 28 SH groups/mole, the results, therefore, indicated that complete reaction had taken place. In a control experiment, aldolase was first treated with excess *N*-ethylmaleimide in 8 M urea, dialyzed, and finally treated with the above sulfonation procedure. The incorporation of radioactivity in this case represents less than 0.1 sulfonate group/mole showing that incorporation occurred specifically at cysteine residues.

*Regeneration of free SH Groups in S-Sulfonated Aldolase.* The $^{35}S$-labeled protein prepared above was treated with β-mercaptoethanol in 4 M urea solution. A portion of the product was then dialyzed exhaustively against water and assayed for protein-bound radioactivity. The remaining portion was diluted to allow the subunits to reassociate and assayed for FDP aldolase activity. Details of these procedures are described in the Methods section.

The dialyzed, thiol-treated protein was found to contain less than 0.1 of $^{35}S$-labeled sulfite group/mole of aldolase. The *S*-sulfonate groups were therefore completely removed from the protein. The enzyme activity of the reconstituted enzyme was 9.5 μmoles of FDP cleaved/min per mg of protein, which is identical with the activity of the native enzyme assayed under the same conditions. In control experiments where S-sulfonated aldolase was treated in exactly the same way except that β-mercaptoethanol was replaced by water, no aldolase activity was detectable even at ten times the usual protein concentration. The high recovery of aldolase activity is entirely reproducible and may be obtained from the S-sulfonated derivative even after several months of storage at 4° as a lyophilized powder. These results suggest that residues in the enzyme which are essential to activity have not been irreversibly modified by the above treatment.

FIGURE 1: Disc gel electrophoretic patterns of S-sulfonated aldolase and CM-aldolase. Left: *S*-CM-aldolase; middle: S-sulfonated aldolase; right: mixture of previous two aldolase derivatives.

*Electrophoretic Pattern of S-Sulfonated Aldolase.* The specific nature and the extent of the above reactions was further shown by examination of the products in disc gel electrophoresis in 8 M urea. As shown in Figure 1, the electrophoretic pattern of S-sulfonated aldolase consists of two bands and is identical with that of *S*-CM-aldolase. The similarity in the electrophoretic migration of the two derivatives was confirmed when the same pattern was obtained by electrophoresis of a mixed sample containing equal amounts of each derivative. This indicates that the total net ionic charge is the same in both cases. At the alkaline pH of the electrophoresis, the CM and the sulfonate groups would both contribute one negative charge per group. This means that the number of sulfonate groups is equal to the number of CM groups and indicates that complete sulfonation has taken place.

The two components in CM-aldolase have been shown to correspond to two carboxymethylated subunits (α and β) which differ in primary structure (Chan *et al.*, 1967; Morse *et al.*, 1967). By analogy, the two components in S-sulfonated aldolase are presumably the sulfonated derivatives of these subunits. The fact that only two components were obtained indicates that side reactions which result in extensive change in the net charge of the protein did not occur.

*The Nature of the Sulfonation Reaction and the Catalytic Requirements.* The incorporation of $^{35}S$-labeled sulfite into aldolase was used to study the various requirements of the reaction. The results are summarized in Table I. Surprisingly, it was found that the addition of either *o*-phenanthroline or copper or both did not affect the extent of the reaction. However, if cysteine was omitted, the amount of incorporation was less than 10%. The role of molecular oxygen was studied by performing the reaction under a nitrogen atmosphere. The extent of the reaction was greatly reduced indicating that oxygen was essential for the reaction. The limited amount of sulfite incorporated was probably due to residual traces of oxygen since in this experiment no attempt was made to remove dis-

TABLE I: Incorporation of $^{35}S$-Labeled Sulfite under Various Conditions.[a]

| Additions | Sulfite Groups Incorpd/Mole | % of Total Protein SH |
|---|---|---|
| None | 27.3 | 98 |
| $CuSO_4$ | 27.5 | 98 |
| $CuSO_4$ + *o*-phenanthroline | 26.5 | 95 |
| Cysteine omitted | 2.3 | 8 |
| Cysteine omitted but with $CuSO_4$ + *o*-phenanthroline | 3.1 | 11 |
| $N_2$ atmosphere[b] | 11.1 | 40 |

[a] The reaction mixture contained dialyzed aldolase (2 mg/ml), Tris-HCl (0.1 M) (pH 8.4), urea (8 M), cysteine ($2 \times 10^{-4}$ M), and $Na_2{}^{35}SO_3$ (0.05 M) (80,000 cpm/$\mu$mole) except where otherwise stated. After incubation at 25° for 1 hr in an open vessel the protein was precipitated with trichloroacetic acid, washed, and radioactivity was determined. [b] A closed Warburg flask was used, and after introducing a nitrogen atmosphere the reaction was started by tipping cysteine into the mixture containing the remaining reagents.

TABLE II: The Effect of Various SH Compounds on the Sulfonation of Aldolase.[a]

| SH Compound Added | Sulfite Groups Incorpd/ Mole | % of Total Protein SH |
|---|---|---|
| None | 2.3 | 8 |
| Cysteine ($2 \times 10^{-4}$ M) | 27.8 | 99 |
| $\beta$-Mercaptoethanol ($2 \times 10^{-4}$ M) | 1.6 | 6 |
| $\beta$-Mercaptoethanol ($2 \times 10^{-3}$ M) | 0.8 | 3 |
| Dithiothreitol ($2 \times 10^{-4}$ M) | 8.2 | 30 |
| $\beta$-Mercaptoethylamine ($2 \times 10^{-4}$ M) | 27.8 | 99 |

[a] The reaction conditions used were identical with those described in Table I except that cysteine was omitted.

solved oxygen completely from the solutions of the reactants.

S-Sulfonated aldolase prepared in the absence of copper and *o*-phenanthroline was compared with the same derivative prepared in the presence of these reagents. The electrophoretic patterns of the two samples were indistinguishable and the recoveries of enzyme activity upon treatment with excess mercaptoethanol were identical. The only difference observed was that after dialysis the protein derivative prepared in the presence of copper still contained significant amounts of copper as shown by color reaction with sodium diethyldithiocarbamate. Since this may be an undesirable contamination in many cases, copper and *o*-phenanthroline were omitted in all subsequent experiments.

*The Effect of Other Sulfhydryl Compounds.* Since cysteine was shown to be necessary for the reaction, other SH compounds were tested for their ability to promote sulfonation. As shown in Table II, there appears to be some specificity in the requirement for a SH compound. $\beta$-Mercaptoethanol was completely ineffective at two different concentrations and dithiothreitol (Cleland's reagent) had only a slight effect on the incorporation. On the other hand, $\beta$-mercaptoethylamine was similar to cysteine in giving a complete reaction. The significance of these results is discussed in a subsequent section.

*Other Properties of the Reaction.* Figure 2 shows the time course of the sulfonation of aldolase. Under the conditions employed, the reaction was complete in 1 hr. The rate of the reaction was found to vary considerably with the extent to which the reaction mixture was in contact with air. For a typical, large-scale preparation using 200 mg of aldolase in 40 ml of 8 M urea solution, it was found necessary to direct a slow stream of air over the mixture with constant stirring.

The effect of varying the concentration of cysteine is presented in Figure 3. Complete incorporation was obtained at cysteine concentrations of $2 \times 10^{-3}$ and $2 \times 10^{-4}$ M. The amount of incorporation then decreased with either higher or lower cysteine concentration. These effects are discussed later in connection with the mechanism of the reaction.

Since the oxidation of SH groups by molecular oxygen is known to be catalyzed by traces of heavy metals, the effect of chelating agents was investigated. The results are given in Table III. EDTA was found to have a partially inhibitory effect at concentrations of $10^{-5}$ M or higher. The effect could be abolished by adding $Cu^{2+}$ or $Mg^{2+}$ ions. On the other hand, sodium diethyldithiocarbamate which is known to bind strongly to copper does not inhibit significantly even at $10^{-2}$ M concentration. Although the participation of trace metals is not entirely ruled out by these results, the fact that EDTA inhibits only at high concentrations suggests that it is of secondary importance.

Table IV shows the effect of pH on the sulfonation reaction. Complete sulfonation was still obtained when the pH was reduced to 7.0. At pH lower than 7.0 the extent of the reaction decreased in agreement with the suggestion that one of the reacting species is $SO_3^{2-}$ (Cecil and McPhee, 1955) and the $pK_2$ of sulfite is about 7. The decrease in incorporation at or above pH 9.5 is interesting in that it suggests that a protonated amino group in cysteine may be necessary for its effect on the reaction.

*Comparison with Other Methods of Sulfonation.* In

TABLE III: The Effect of EDTA and Sodium Diethyldithiocarbamate on the Sulfonation of Aldolase.[a]

| Additions | Sulfite Groups Incorpd/ Mole | % of Total Protein SH |
|---|---|---|
| None | 27.5 | 98 |
| EDTA ($10^{-4}$ M) | 26.8 | 96 |
| EDTA ($10^{-3}$ M) | 20.2 | 72 |
| EDTA ($10^{-2}$ M) | 11.5 | 41 |
| EDTA ($10^{-2}$ M) + $CuSO_4$ ($10^{-2}$ M) | 27.0 | 96 |
| EDTA ($10^{-2}$ M) + $MgSO_4$ ($10^{-2}$ M) | 27.2 | 97 |
| Sodium diethyldithiocarbamate ($10^{-4}$ M) | 27.2 | 97 |
| Sodium diethyldithiocarbamate ($10^{-2}$ M) | 24.8 | 89 |

[a] The reaction conditions were identical with those described in Table I.

preliminary experiments, it was found that little or no aldolase activity could be recovered from S-sulfonated aldolase prepared by the cupric ammonium sulfite method (Swan, 1957) and by the use of tetrathionate in combination with sulfite (Bailey and Cole, 1959; Katsoyannis *et al.*, 1967). However, previous workers have used these methods successfully to prepare the S-sulfonated derivatives of the A and B chains of insulin (Dixon and Wardlaw, 1960; Katsoyannis *et al.*, 1967) and insulin activity was obtained from these derivatives after treatment with excess thiol and reoxidation. The extent of recovery of biological activity is important in many applications of the sulfonation reaction. It was, therefore, of interest to compare the above-mentioned methods using as a criterion the subsequent recovery of aldolase activity under identical conditions. The results are shown in Table V. In two separate experiments, no aldolase activity was recovered after using the tetrathionate–sulfite method. The failure to obtain activity was probably not due to the effect of substituting guanidine hydrochloride (8 M) for urea, since when the method described in this paper was applied in guanidine hydrochloride (8 M), 80% recovery was obtained. The cupric ammonium sulfite method was found to lead to the recovery of only a small fraction of the original activity. Thus, in the case of aldolase the method described in this paper is far superior in the extent of recovery of activity.

TABLE IV: The Effect of pH on Sulfonation.[a]

| Buffer Used (pH) | % of Total Protein SH |
|---|---|
| Sodium acetate (5.0) | 26 |
| Sodium citrate (6.0) | 47 |
| Sodium phosphate (7.0) | 98 |
| Sodium phosphate (7.5) | 97 |
| Tris-HCl (7.5) | 102 |
| Tris-HCl (8.4) | 102 |
| $NaHCO_3$–$CO_2$ (9.5) | 21 |
| $NaHCO_3$–$CO_2$ (10.0) | 15 |

[a] The reaction conditions were identical with those described in Table I except for changes in the buffer used. The buffer concentration was 0.1 M in all experiments.

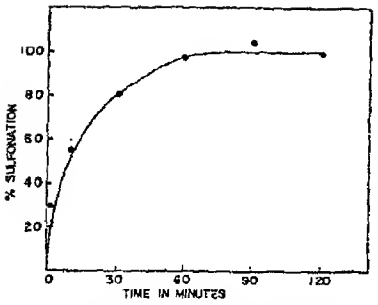


FIGURE 2: Time course of the sulfonation of aldolase. The incorporation of 28 sulfite groups/mole of aldolase is taken as 100%. The reaction conditions were as described in Table I. At various times indicated, aliquots were removed and added to 10% trichloroacetic acid to stop the reaction. Incorporation was determined by the Millipore method (see Materials and Methods).

*Application of the Method to Other Proteins.* In order to test if the above sulfonation method was generally applicable, two other purified proteins were similarly sulfonated. In one experiment lactate dehydrogenase (1.1.1.27) from rabbit muscle was used. The commercial preparation, which contained mainly the $M_4$ isozyme with small amounts of the $M_3H$ hybrid, was passed through a Sephadex G-25 column to remove $(NH_4)_2SO_4$.

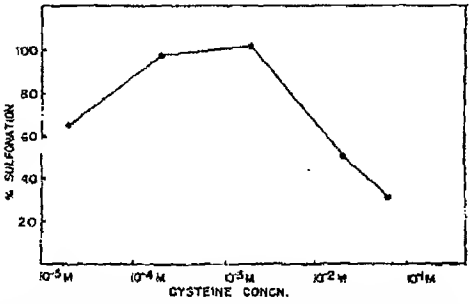


FIGURE 3: The effect of varying cysteine concentration on the extent of sulfonation of aldolase. The details described in the legend to Figure 2 also apply here except that cysteine concentration is varied.

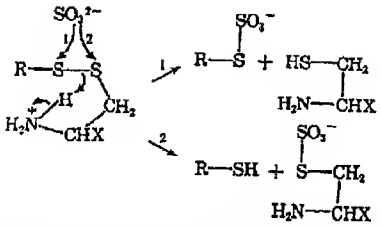

FIGURE 4: A possible explanation of the effect of cysteine and β-mercaptoethylamine. R represents protein and X = $CO_2$ or H for cysteine and β-mercaptoethylamine, respectively.

This sample was sulfonated with radioactive sulfite as described for aldolase.

The incorporation was 0.119 μmole of sulfite/mg of protein or 16.1 equiv for a molecular weight of 135,000 daltons. Other workers (Di Sabato *et al.*, 1963; Pfleiderer *et al.*, 1959) have reported the presence of 16 SH groups/mole of the enzyme and the absence of disulfide bonds. The above data therefore show that lactate dehydrogenase was completely sulfonated by this method.

Commercial pepsinogen was similarly treated with $^{35}S$-labeled sulfite. The incorporation of 5.8 groups of sulfite/mole again indicates complete reaction since the molecule of pepsinogen is known to contain 3 disulfide bonds (Arnon and Perlmann, 1963). These results suggest that the method should be applicable to the sulfonation of proteins in general. In this method, the native configuration of the protein may be regarded as unimportant since the reaction takes place in dissociating medium.

## Discussion

It is evident from the above results that the SH groups of aldolase are rapidly and completely sulfonated when treated with sodium sulfite and catalytic amounts of cysteine in 8 M urea solution. Three lines of evidence indicate that the reaction is specific for SH groups. (1) The number of sulfonate groups incorporated is equal to the number of SH groups known to be present in aldolase. (2) All of these sulfonate groups are removed upon treatment with excess thiol. (3) The electrophoretic pattern of the product is identical with that of CM-aldolase, as would be expected from a consideration of the net charge in these derivatives. Although modification of other residues under the reaction conditions cannot be ruled out, there appears to be no sulfonation of residues other than cysteine since *N*-ethylmaleimide-treated aldolase did not incorporate significant radioactivity when similarly treated with $^{35}S$-labeled sulfite. Carbamylation is unlikely to occur to a significant extent since urea solutions freshly prepared from recrystallized urea are known to be relatively free from cyanate (Stark *et al.*, 1960). The complete recovery of enzyme activity suggests that residues which are essential for enzyme activity have remained intact.

The reaction requires the addition of certain SH compounds and the presence of molecular $O_2$ and thus indicates that the reaction takes place *via* the formation of disulfide bonds and subsequent sulfitolysis. Although the oxidation of SH compounds in aqueous solutions by molecular $O_2$ has been much studied, the mechanism of the reaction is far from clear (Cecil and McPhee, 1959). The system studied here is further complicated in that the formation of protein–protein disulfide bonds, protein–cysteine-mixed disulfide, and cysteine are all possible. At any moment, during the reaction, all these components react reversibly with sulfite, unoxidized cysteine, and protein SH. No attempt is therefore made to formulate precisely the course of the reaction on the basis of available data. However, since the initial presence of cysteine is required, the reaction clearly does not proceed mainly *via* protein–protein disulfides as intermediates. The extensive formation of these bonds in aldolase subunits which have a molecular weight of approximately 40,000 daltons (Kawahara and Tanford, 1966; Sia and Horecker, 1968) will encounter considerable steric hindrance.

The reaction is likely to involve the initial formation of cystine and the mixed disulfide between cysteine and protein SH. Subsequent sulfitolysis then gives cysteine sulfonate and the S-sulfonated protein. The observation that cysteine can be replaced by β-mercaptoethylamine but not by β-mercaptoethanol or dithiothreitol indicates a role for the neighboring amino group. The decrease in the extent of the reaction at pH 9.5 further suggests that the amino group must be protonated. It has been reported previously (McPhee, 1956) that disulfide molecules containing a positive charge undergo much faster sulfitolysis. In the case of the mixed disulfide between protein SH and cysteine, a possible explanation is presented in Figure 4. The reaction proceeds *via* the nucleophilic attack of the $SO_3^{2-}$ ion on one of the sulfur atoms. This attack is facilitated by the ability of the displaced sulfur atom to pick up a proton immediately from the neighboring amino group. Since aldolase was shown to undergo rapid sulfonation, it is possible that in Figure 4

TABLE V: Recovery of Aldolase Activity from S-Sulfonated Aldolase.[a]

| Method of Sulfonation | % Recov of Aldolase Act. |
|---|---|
| Tetrathionate+sulfite in guanidine hydrochloride[b] | 0 |
| Cupric ammonium sulfite in 8 M urea[c] | 22 |
| Cysteine+$O_2$+sulfite in 8 M urea[d] | 100 |
| Cysteine+$O_2$+sulfite in guanidine hydrochloride[d] | 80 |

[a] Aldolase was sulfonated at 1 mg/ml in each case. The products were dialyzed and then treated as described in Methods for the regeneration of enzyme activity. [b] Katsoyannis *et al.* (1967). [c] Swan (1957). [d] As described in this paper.

reaction 1 takes place in preference to reaction 2. However, there are no obvious mechanistic reasons for such preference and an examination of molecular models has failed to reveal any steric reasons. Kinetic studies using structural analogs of cysteine are being conducted in this laboratory in order to clarify the reaction mechanism.

The proposed role of cysteine is in agreement with the effect of varying cysteine concentration shown in an earlier section. At low cysteine concentrations, the formation of mixed disulfide is limited and protein sulfonation is therefore incomplete. On the other hand at concentrations higher than the optimal range, a considerable amount of cysteine remains unoxidized at the end of the incubation period thus tending to reverse the reaction.

Previous work (Stellwagen and Schachman, 1962) has shown that aldolase subunits exist as "markedly disorganized" polypeptides in solutions containing $\geq 4$ M urea. Thus, during S sulfonation and the subsequent removal of sulfonate groups both of which take place in 8 M urea, the aldolase polypeptides assume configurations essentially different from its native structure. The introduction of 28 negative charges of considerable bulk must be expected to lead to a further departure from the native state especially since aldolase has been reported to contain 7–8 (Kowal *et al.*, 1965) or 16 (Stellwagen and Schachman, 1962) "buried" SH groups. That the native enzyme was obtained after reconstitution can be inferred from previous work using similar conditions (Stellwagen and Schachman, 1962) and is supported by the complete recovery of activity. Thus, there appears to be a spontaneous refolding of the aldolase polypeptides into the native configuration. This represents one of an increasing number of cases (Anfinsen and Haber, 1961; Deal *et al.*, 1963b) where proteins have been reconstituted from a denatured state. The result is consistent with the hypothesis that the native structure of a protein is determined only by its amino acid sequence (Crick, 1958).

The sulfonation reaction was applied by Dixon and Wardlaw (1960) in an elegant separation of the A and B chains of insulin. The use of the reaction is, however, not restricted to the cleavage of disulfide bonds but may include the reversible blocking of SH groups. An important consideration in many such applications is the recovery of biological activity. Hitherto complete sulfonation of proteins was attained by using either cupric ammonium hydroxide at pH 10 (Swan, 1957) or sodium tetrathionate (Bailey and Cole, 1959) as oxidizing agents. As shown in this paper, the recovery of aldolase activity after exposure to these sulfonation procedures is low. In contrast, the method described here gave a complete recovery of aldolase activity. It remains to be determined if such differences are shown when the methods are applied to other proteins. The reasons for these differences are not known. A previous report (Bailey and Cole, 1959) indicates that there may be some modification of tryptophan residues when protein is treated with tetrathionate. At the alkaline pH of the cupric ammonium hydroxide method, some deamidation or peptide hydrolysis may occur. The method described in this paper thus represents an alternative procedure which may have considerable advantages over existing methods.

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References

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.
Arnon, R., and Perlmann, G. E. (1963), *J. Biol. Chem. 238*, 653.
Bailey, J. L., and Cole, R. D. (1959), *J. Biol. Chem. 234*, 1733.
Baranowski, T., and Niederland, T. R. (1949), *J. Biol. Chem. 180*, 543.
Bücher, T. (1947), *Biochim. Biophys. Acta 1*, 292.
Cecil, R., and McPhee, J. R. (1955), *Biochem. J. 60*, 496.
Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14*, 255.
Chan, W., Morse, D. E., and Horecker, B. L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1013.
Cole, R. D. (1967), *Methods Enzymol. 11*, 206.
Crick, F. H. C. (1958), *Symp. Soc. Exptl. Biol. 13*, 138.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Deal, W. C., Rutter, W. J., Massey, V., and Van Holde, K. E. (1963b), *Biochem. Biophys. Res. Commun. 10*, 49.
Deal, W. C., Rutter, W. J., and Van Holde, K. E. (1963a), *Biochemistry 2*, 246.
Di Sabato, G., Pesce, A., and Kaplan, N. O. (1963), *Biochim. Biophys. Acta 77*, 135.
Dixon, G. H., and Wardlaw, A. C. (1960), *Nature 188*, 721.
Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., and Trakatellis, A. C. (1967), *Biochemistry 6*, 2635.
Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.
Kobashi, K., and Horecker, B. L. (1967), *Arch. Biochem. Biophys. 121*, 178.
Kowal, J., Cremona, T., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 2485.
McPhee, J. R. (1956), *Biochem. J. 64*, 22.
Morse, D. E., Chan, W., and Horecker, B. L. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 628.
Pfleiderer, G., and Jeckel, D. (1957), *Biochem. Z. 329*, 370.
Pfleiderer, G., Jeckel, D., and Wieland, T. (1959), *Arch. Biochem. Biophys. 83*, 275.
Racker, E. (1947), *J. Biol. Chem. 167*, 843.
Sia, C. L., and Horecker, B. L. (1968), *Arch. Biochem. Biophys. 123*, 186.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Stellwagen, E., and Schachman, H. K. (1962), *Bio-*

*chemistry 1*, 1056.
Swan, J. M. (1957), *Nature 180*, 643.
Swenson, A. D., and Boyer, P. D. (1957), *J. Am. Chem. Soc.* 79, 2174.
Taylor, J. F., Green, A. A., and Cori, G. T. (1948), *J. Biol. Chem. 173*, 591.

# Spectral Studies of Iron Coordination in Oxidized Compounds of Hemoproteins. Difference Spectroscopy below 250 mμ*

Arthur S. Brill† and Howard E. Sandberg‡

ABSTRACT: The feasibility of observing the spectral behavior of protein groups in the coordination sphere of the iron in hemoproteins has recently been demonstrated. Application of the method, difference spectroscopy below 250 mμ, to oxidized compounds of hemoproteins is reported in this paper. The absolute absorption spectrum (250–450 mμ) of bacterial catalase compound I is displayed. Ultraviolet difference spectra (210–280 mμ) of compounds III of metmyoglobin and methemoglobin and compounds I and II of bacterial catalase and horseradish peroxidase *vs.* the free hemoproteins are shown. Compounds III of metmyoglobin and methemoglobin, and compounds II of bacterial catalase and horseradish peroxidase have one difference band which peaks in the region 237–247 mμ. Absorption in this region is assigned, in part, to a transition involving histidine in the fifth coordination position, and in the other part to a charge transfer transition involving porphyrin. Compound I of bacterial catalase has two difference bands (235 and 224 mμ), while I of horseradish peroxidase has a broad band which is probably the sum of two bands. The spectral properties of compounds I are like those of ferric hemoprotein complexes. The spectral properties of compounds II and III support the other evidence that these are ferryl structures.

Several years ago, in the course of investigating possible involvement of tyrosine in the formation of compound I of catalase, we recorded difference spectra *vs.* free enzyme down to a wavelength of 250 mμ and noted that the absorbance rose just as this wavelength was approached. We were thereby prompted to look deeper into the ultraviolet region, and found the bands described in this paper. In checking possible explanations of these bands, we were led to try more fundamental experiments with hemoprotein complexes (Brill and Sandberg, 1968). In the latter research, the groups involved have been identified on the basis of data from simple ligands. In this paper, the difference spectra of peroxide compounds *vs.* free proteins are reported and used to describe with more assurance than previously the coordination spheres of the iron in the oxidized compounds of MetMb,[1] MetHb, BMC, and HRP.

Only one spectroscopically distinct compound (denoted in the literature as "compound III") has been observed upon reaction of MetMb with any of the substrates $H_2O_2$, methyl hydroperoxide, or ethyl hydroperoxide. The stoichiometry of reduction of compound III by ferrocyanide indicates that this compound has one oxidizing equivalent above free MetMb (George and Irvine, 1952, 1953). Since the peroxide substrate has two oxidizing equivalents, one of the equivalents is not retained by the heme group.

Similarly, only one spectroscopically distinct compound (also called "III") has been observed for the reaction of MetHb with $H_2O_2$ (Keilin and Hartree, 1951; Dalziel and O'Brien, 1954). The oxidation state of MetHb III has not yet been determined. It is unlikely to differ from that of MetMb III.

The reaction of HRP with either $H_2O_2$ (concentration less than 1 mM so that a third compound of peroxidase does not form) or alkyl hydroperoxides produces two spectroscopically distinct compounds, labeled "I" and "II" on the basis of order of appearance. The stoichiom-

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[1] Abbreviations used: MetMb, horse heart ferrimyoglobin; MetHb, horse ferrihemoglobin; BMC, bacterial micrococcus catalase; HRP, horseradish peroxidase; HBC, horse blood catalase; suffixes I, II, and III, compounds I, II, and III.

#3

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# Reversible protection of disulfide bonds followed by oxidative folding render recombinant hCGβ highly immunogenic

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**Abstract**

Active immunization of women against human chorionic gonadotropin (hCG) has been considered as a promising option for contraception. However, prototype hCG vaccines based on natural sources of antigen are expected to be costlier for use by common people. In the present report, a functionally active, cost-effective antigen of bacterial origin has been described. Sulfonation of thiol groups of the protein, anion-exchange purification, refolding with concomitant formation of disulfide bonds in the presence of cysteamine–cystamine redox buffer, and slow removal of denaturant resulted in 95% homogenous, monomeric form of the antigen. The recombinant processed antigen [CGβ(p)] obtained this way was highly immunopotent. Cellular DNA and endotoxin contaminants were appreciably low in the final product. The immunogenic response was drastically reduced with the unprocessed antigen. This finding envisages better prospect of a cost-effective hCG vaccine for birth control. 

*Keywords:* Immunogenicity; Refolding; Recombinant CGβ

## 1. Introduction

Human chorionic gonadotropin (hCG) is a heterodimer composed of an alpha (hCGα)- and a beta (hCGβ)-subunit that combine non-covalently to form a biologically active hormone. It is an early signal for conception, and considered essential for both establishment and maintenance of pregnancy. Therefore, active immunization of women against hCG has been considered as a promising option for contraception. The rationale for using a vaccine against hCG is to maintain sufficient circulating antibodies capable of binding to hCG, rendering them ineffective for biological activities [1,2].

So far, three prototype hCG vaccines based on full-length native hCGβ (nhCGβ) and synthetic peptide consisting of 37 amino acid residues of the C-terminus of nhCGβ conjugated to tetanus or diphtheria toxoid as a carrier protein have been subjected to clinical trials [3–5]. However, none of these prototype vaccines is suitable in its present form for manufacture and widespread application for population control, mainly due to unaffordable cost. Hence, there is a need to produce a cost-effective immunogen by recombinant DNA technology to meet this challenge [6]. During the last decade, several mammalian systems [7–10] have been tried for over-expression of a functional hCGβ. However, the products were unsuitable due to quantitative and qualitative reasons.

A cost-effective vaccine may be expected, if quality antigen is produced in high quantities in suitable microbial systems. Bacterial expression of hCGβ, its folding and assembly with counter subunit, has been reported earlier in a different context [11]. In a previous paper [12], for the first time it was shown that

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bacterially produced hCGβ evokes a significant immune response in rats and monkeys. Here, purification and refolding of hCGβ in an immunologically active form that consistently elicits high antibody titre in rats and partial characterization of the antigen are reported.

## 2. Materials and methods

### 2.1. Culture of transformed E. coli expressing hCGβ

*E. coli* strain BL21λDE3 [13], transformed with a plasmid harbouring gene of hCGβ under the control of a phage T7 promoter, was grown in a 14L bioreactor (Chemap AG, Switzerland). The composition of the medium and the culture conditions have been described earlier [14]. The culture was grown until the cell density of 6 $gl^{-1}$ (dry weight) was attained. It was then induced with 3 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. During fermentation the temperature, pH and dissolved oxygen were maintained at 37.0 ± 0.5°C, 7.2 to 7.4 and 20 ± 5%, respectively. The expression of hCGβ at the end of the induction period was confirmed by Western blot analysis of the cell lysate.

### 2.2. Isolation of inclusion bodies

Prior to cell separation, the culture broth was treated with phenol and chloroform to a final concentration of 0.5% (v/v) at 37°C for 1 h. After centrifugation the cell pellet was suspended in 50 mM Tris Cl buffer (pH 8.0) containing 5 mM EDTA, and disrupted in a French Press (SLM Instruments, Urbane, IL) at 1000 kg $cm^{-2}$ pressure [15]. The inclusion bodies were recovered by centrifugation at 7000 *g* for 10 min, and washed three times in the same buffer. The inclusion body pellet was stored at 4°C and processed within 2 days.

### 2.3. Purification of recombinant hCGβ

The inclusion body pellet was solubilized (1 gm pellet per 20 ml buffer) in 50 mM Tris Cl (pH 7.5)–6 M guanidine Cl buffer. The solution was clarified by centrifugation at 35,000 *g* for 45 min at 15°C. The thiol groups of hCGβ were protected by sulfonation through treatment with sodium sulfite (10 mg $ml^{-1}$) and sodium tetrathionate dihydrate (5 mg $ml^{-1}$) at room temperature for 12–14 h. The sulfonated protein (Protein-$SSO_3$) was dialysed in 50 mM Tris Cl buffer (pH 7.0)–2 M urea, and purified on a Q-Sepharose column (Pharmacia, Uppsala, Sweden). The protein was subsequently eluted by step-gradient of NaCl, and protein fraction between 12 to 21 mS $cm^{-1}$ conductivity was collected. In order to minimize cellular DNA and endotoxin contamination in the final product, the eluted protein was recycled three consecutive times on a Q-Sepharose column equilibrated with Tris–urea–NaCl buffer at 19 mS $cm^{-1}$ conductivity.

### 2.4. Refolding of sulfonated hCGβ

Prior to refolding, the sulfonated hCGβ was dialysed in 50 mM Tris Cl (pH 7.5) containing 3 M urea, diluted to the concentration of 120 μg $ml^{-1}$ and degassed. In vitro folding of the protein was carried out in a redox buffer containing reduced cysteamine-oxidized cystamine. The folding of hCGβ was conducted at 5–8°C for a period of 12 h. Finally, the urea was removed by step-wise dialysis in buffers containing progressively lower concentrations of urea (3 M → 2 M → 1.5 M → 1 M → 0.5 M → 0 M). Enzyme-linked immunosorbent assay (ELISA) using native hCGβ specific monoclonal antibody was employed to compare antigens obtained in different redox buffers. The antigen processed by this route was termed as CGβ(p). In an alternate procedure, Q-Sepharose purified non-sulfonated protein was simply refolded by removal of urea in dialysis against normal saline. The protein folded in this manner was termed CGβ(up).

### 2.5. Detection of cellular DNA and endotoxin

The presence of cellular DNA, if any, in the purified antigen was determined by DNA slot-blot hybridization. For DNA probe, a mixture of purified host (*E. coli* strain BL21λDE3) chromosomal and plasmid DNA was labeled with $^{32}$[P]-dCTP by nick translation using NE Blot Kit (NEB Laboratories, UK). Threefold serial dilutions of the same DNA mixture (900 pg–3.7 pg) as standard and unknown samples of different dilutions were blotted on a Hybond membrane (DuPont, Bannockburn, IL). The membrane was processed and hybridized using the standard protocol [16]. The hybridized membrane was exposed to a PhosphorImager screen for 2 h and analysed (GS-250, Bio-Rad Laboratories, California). A standard curve was plotted with different counts (cpm) versus corresponding standard DNA samples in pg. The DNA quantity in the unknown sample was then determined from the standard curve.

The amount of endotoxin present in the final product was determined by a limulus amoebocyte lysate (LAL) test based on gel clot assay using Endosafe kit (Charles River, Charleston). In brief, samples of different dilutions (360 ×, 720 ×, 1440× and 2880×) were incubated with the reagent at 37°C for 1 h. The presence of endotoxin in the sample was indicated by the formation of an opaque gel. A firm gel is formed as the concentration of endotoxin exceeds the sensitivity

of the reagent. Thus the concentration of endotoxin in the original sample was calculated by multiplying the sensitivity of the test (0.25 EU $ml^{-1}$) with the dilution factor.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

Immunoreactivity of the antigen was assayed by ELISA as described earlier [17]. Briefly, a 96-well plate was coated (triplicate wells) with either native or recombinant antigen [nhCGβ and CGβ(p)] at a concentration of 5 μg $ml^{-1}$ for 1 h at 37°C. The antigen-coated wells were blocked with 1% BSA for 1 h and reacted with serially diluted monoclonal antibody against native hCGβ for 1 h at 37°C. The plate was washed four times in phosphate-buffered saline Tween 20 (PBST), and bound antibody was detected by incubating with 1:10,000 diluted goat anti-mouse IgG-HRP conjugate (NII Reagent Bank, New Delhi, India) for 1 h at 37°C. The plate was thoroughly washed with PBST and then colour was developed by reacting with 3,3'-diaminobenzidine (DAB) and $H_2O_2$ in citrate–phosphate buffer for 5 min. The reaction was stopped by adding 5 N $H_2SO_4$, and the magnitude of reaction was determined by an ELISA reader.

### 2.7. Biological activity of CGβ(p)

An equal molar proportion of CGβ(p) and nCGα mixture was incubated at 37°C for 16 h. The annealed heterodimer, so formed, was quantitated by radioimmunoassay (RIA). The capacity of the annealed heterodimer to stimulate testosterone (T) production was determined by mouse Leydig cell bioassay as described [18]. Leydig cells were prepared from mouse testes and were incubated with various concentrations of heterodimer and nhCG. The amount of T released by the cells was assayed by a standard World Health Organization (WHO) protocol using reagents supplied by WHO.

### 2.8. Immunization and immunoassay of antisera

The immunization protocol consisted of a primary immunization followed by two boosters in the 4th and 8th weeks. Four groups, each comprising six female Wistar rats 4–6 week old were used in the study. The animals of the first three groups received intramuscular injections of a 10 μg equivalent CGβ(p) produced in three different batches, and the fourth group received CGβ(up). The antigens were adsorbed on aluminium hydroxide gel (Alhydrogel, Superfos Biosector a/s, Denmark) as an adjuvant. Rats were bled on the 9th week of immunization. The anti-hCG antibody titre of sera was assayed by using a hCG-dimer specific radioimmunoassay [19].

### 2.9. Mixed lymphocytes proliferation assay

Mononuclear cells from heparinized peripheral blood of monkeys immunized with CGβ(p) were used for the assay. The mononuclear cells were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the low density cells were collected and washed in RPMI-1640. The mononuclear cells were cultured using RPMI-1640 containing 10% autologous sera. About $5 \times 10^5$ cells in 200 μl were grown in triplicate wells of a 96-well flat-bottom microtitre plate for 54 h in the presence of either 10 μg $ml^{-1}$ nhCGβ or CGβ(p). Three replicate wells were also set up, either with medium (no antigen) or with 5 μg $ml^{-1}$ Concanavalin-A as controls. After 54 h of culture, cells were pulsed with 1 μCi $^3$[H]-thymidine for 18 h. The pulsed cells were harvested, and the amount of labeled thymidine incorporation in each sample was determined by a liquid scintillation counter (Beckmann, Palo Alto, CA).

### 2.10. Analytical

The protein concentration was estimated by BCA reagent (Pierce, Rockford, IL). The purity of the antigen was determined in 12.5% (w/v) SDS–PAGE [20]. The hCGβ was identified by Western blot, where resolved protein in the polyacrylamide gel was electrotransferred on a nitrocellulose membrane [21], and probed with hCGβ-specific monoclonal antibody. N-terminus sequence of the purified recombinant hCGβ was determined in an automatic sequencer (Applied Biosystem, California). The heterogeneity of CGβ(p) was determined by non-reducing SDS–PAGE and by HPLC–GPC using an analytical Protein-Pak SW 300 column (Waters, Japan). The flow rate of the mobile phase (100 mM phosphate buffer, pH 6.8) in column was maintained at 1 ml $min^{-1}$. The intrinsic and extrinsic fluorescence spectra of CGβ(p) were studied as per protocol described earlier [12].

## 3. Results

### 3.1. Fermentation and isolation of inclusion bodies

The transformed *E. coli* was grown in a 14L bioreactor under controlled pH, temperature and concentration of dissolved oxygen. The medium was periodically supplemented with minerals and complex proteinaceous nutrients. The amount of hCGβ expressed in the IPTG induced cells was 10% of the total stainable protein, calculated on the basis of densitometric analysis of the Coomassie brilliant blue stained gel (data not shown). The total yield of hCGβ in the culture stage was 150 mg $l^{-1}$. The purity of

hCGβ in the washed inclusion body pellet was 30–35% [15]. The inclusion body pellet was solubilized in 6 M guanidine Cl, and the thiol groups of the protein were sulfonated to avoid the formation of aberrant intrachain and interchain disulfide bonds, and subsequent aggregation. Each thiol group was converted to sulfonated form, as no free thiol group was detected in the modified protein.

### 3.2. Purification of sulfonated hCGβ

Sulfonated hCGβ was purified using a fast flow Q-Sepharose matrix. In each run 120 mg sulfonated protein was loaded on the column, of which 60 ± 6% protein was bound to the matrix. The sulfonated hCGβ was eluted in a step gradient between 0.12 to 0.25 M concentrations of NaCl, which correspond to the conductivity of 12 to 21 mS $cm^{-1}$ (Fig. 1). The purified hCGβ was more than 87% homogenous on SDS–PAGE, and immunoreactive to hCGβ-specific monoclonal antibody as shown in the Western blot (Fig. 1, inset). The recovery efficiency of the anion-exchange purification step was 90 ± 4% (number of experiments, $n$ = 3). The partial N-terminal sequence of Q-Sepharose purified sulfonated hCGβ was SKEPLRPRCRPINATLAVEK, the same as the first 20 amino acids of the nhCGβ.

### 3.3. Refolding of hCGβ

In vitro refolding of hCGβ with concomitant disulfide bond formation includes regeneration of native, non-covalent interactions and the formation of covalent bonds. In this study, the folding of sulfonated hCGβ was carried out in the presence of different compositions of the cysteamine–cystamine redox pair. After disulfide exchange, urea was removed from the reaction mixture by slow dialysis. Fig. 2 depicts the extent of recognition of CGβ(p), folded in five different compositions of the redox pair, by monoclonal antibody specific to native hCGβ. The antibody recognition to CGβ(p) was minimum at a lower reduction state of the buffer (lower ratios of [Cysteamine] × [Cystamine]$^{-1}$). The antigen–antibody reaction was observed highest at a redox pair composition of 2:1, yet comparable to that with the nhCGβ (Fig. 2). The

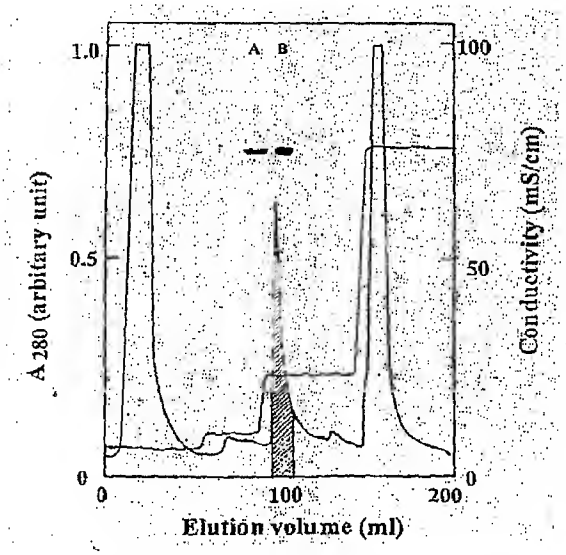


Fig. 1. Purification of sulfonated hCGβ in anion-exchange chromatography. The sulfonated protein was dialysed in 2 M urea–50 mM Tris Cl buffer (pH 7.0), and loaded on a pre-equilibrated Q-Sepharose column at a flow rate of 3 ml $min^{-1}$. After washing the column, the bound protein was eluted by step-gradient of NaCl. The shaded portion of the chromatogram represents elution profile of the sulfonated hCGβ during step-gradient of 0.12 to 0.25 M NaCl. Inset A: Coomassie blue stained band of sulfonated hCGβ; B: Western blot of the protein probed with hCGβ-specific monoclonal antibody.

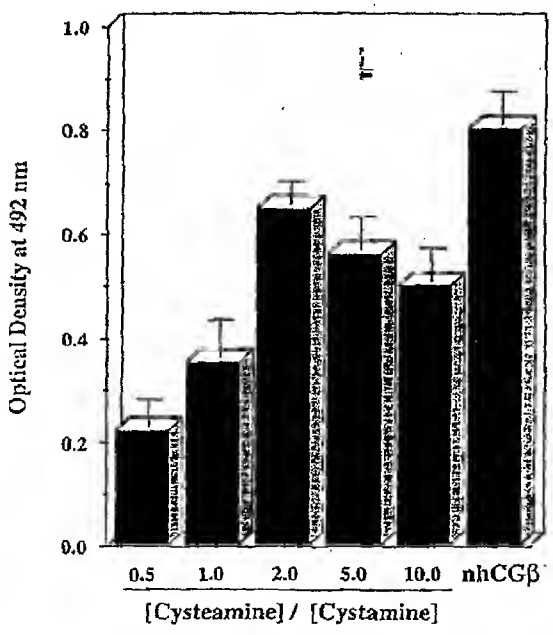


Fig. 2. Immunoreactivity of recombinant antigen, folded in a different composition of redox buffers. In each case 120 μg $ml^{-1}$ degassed sulfonated hCGβ was refolded in the presence of reduced cysteamine-oxidized cystamine at molar proportions of 0.5:1, 1:1, 2:1, 5:1 and 10:1 in 3 M urea–Tris Cl buffer (details are given in Materials and Methods). A 96-well plate was coated with either nhCGβ, or refolded CGβ(p) at a concentration of 5 μg $ml^{-1}$. The coated antigen was probed with 1:10,000 diluted nhCGβ-specific monoclonal antibody. Bar represents the mean ± SEM.

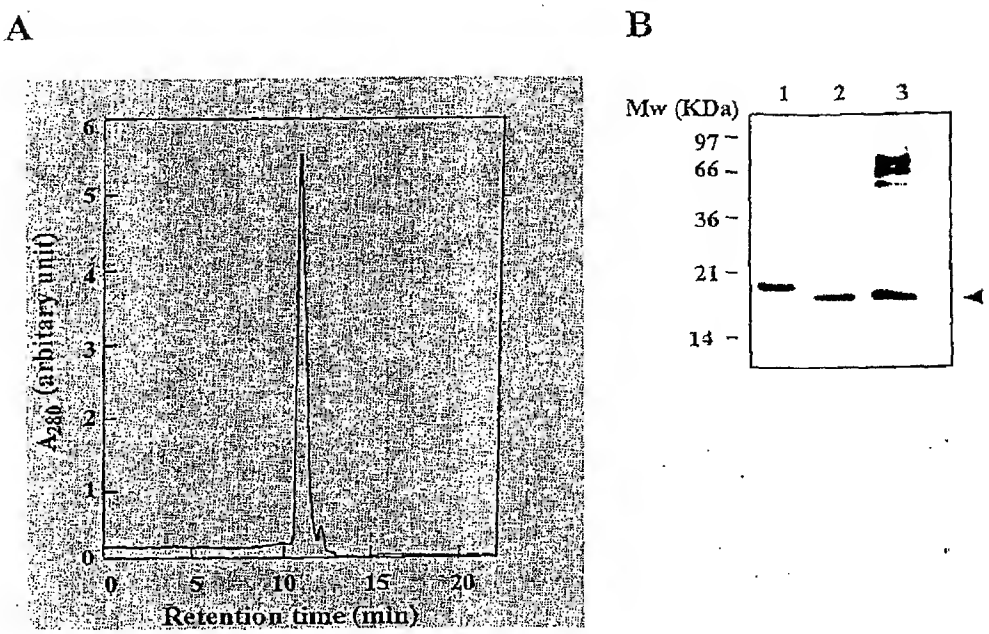


Fig. 3. (A) Retention profile of CGβ(p) in an analytical gel filtration column. The sulfonated hCGβ was refolded in cysteamine–cystamine redox buffer of 2:1 molar composition. The CGβ(p) was loaded on the column and chromatographed using 100 mM phosphate buffer as mobile phase at a flow rate of 1 ml $min^{-1}$. (B) Electrophoresis of CGβ(p) in 12.5% SDS–PAGE gel. Lane 1: CGβ(p) boiled in reducing sample buffer. Lane 2: Freshly refolded CGβ(p) boiled in non-reducing sample buffer. Lane 3: CGβ(p) was frozen and stored prior to boil in non-reducing sample buffer.

CGβ(p) used in the subsequent experiments was refolded in redox buffer composition of 2:1.

In order to determine the formation, if any, of dimer or oligomers during the folding process, CGβ(p) was analysed by HPLC and non-reducing SDS–PAGE. Shown in Fig. 3(A) is a single major peak of monomeric CGβ(p) at 95% homogeneity. The peak retention time of CGβ(p) was 11 min. The same refolded molecule exhibited a single band on gel, which migrated more than the reduced form of the antigen (Fig. 3(B), lane 2). Storage of CGβ(p) in a frozen state facilitates aggregation of the protein, as multimeric forms were detected in the non-reducing gel (Fig. 3(B), lane 3).

The biological activity of CGβ(p) was assayed to find out the magnitude of its native conformation obtained during folding. It was revealed from X-ray crystallography study that hCG-heterodimer is stabilized by a segment of β-subunit which wraps around the α-subunit and is covalently linked by the disulfide bond ($cys_{26}$–$cys_{110}$) [22]. This feature appears to be essential for the association of subunits and also for receptor binding of the hormone. Thus before association it is important for each subunit to attain its native conformation. In the present context, only the natively folded CGβ(p) molecules associate with their counter subunits (nCGα) to become biologically active for stimulating T production in Leydig cells. Therefore, T production has been considered a satisfactory functional parameter to determine the biological activity of this hybrid hormone, which apparently increases with the heterodimeric form and hence with the native conformation of CGβ(p). It was found from the dose response study (data not shown) that 6.7 pg of nhCG or an equivalent amount of the annealed hybrid hormone (based on RIA estimation) stimulated 50 pg testosterone in the Leydig cells. Thus, on the basis of steroidogenesis, the refolding efficiency of CGβ(p) was calculated as 13% (Table 1).

### 3.4. Cellular DNA and endotoxin

Table 2 shows residual cellular DNA and endotoxin in the semiprocessed and in the final product of a representative batch of antigen preparation. It has been found that the majority of DNA and endotoxin loads in the product were removed in the first stage of recycle. In the course of subsequent stages of recycle, these impurity loads in the product were further marginalized to variable extents.

Table 1
Refolding efficiency of CGβ(p)[a]

| Sulfonated-hCGβ (μg ml$^{-1}$) | hCG-equivalent by RIA (μg ml$^{-1}$) | hCG-equivalent by steroidogenesis (μg ml$^{-1}$) | Refolding efficiency (%) |
|---|---|---|---|
| 120 | 15.4 | 15.4 | 13 |

[a] The concentration of sulfonated hCGβ was determined by BCA reagent. The refolded CGβ(p) was annealed with nCGα, and the heterodimer was quantitated by RIA. Based on RIA values, equal amounts of heterodimer and nhCG were used in the dose response study. The T produced by the Leydig cells was measured by WHO kits. The dose response curves for nhCG and that of heterodimer were superimposable, which means heterodimer detected by RIA was 100% biologically active. So, the refolding efficiency was calculated as 15.4/120 (=13%), as 1 mol of CGβ(p) is annealed with 1 mol of nCGα to form 1 mol of biologically active hormone.

Table 2
DNA and endotoxin contents in the intermediate and final product

| Sample type | Protein (mg ml$^{-1}$) | DNA (pg ml$^{-1}$) | Endotoxin (EU ml$^{-1}$) | DNA in unit protein (pg mg$^{-1}$) | Endotoxin in unit protein (EU mg$^{-1}$) |
|---|---|---|---|---|---|
| Initial[a] | 1.23 | 1965 | > 360 | 1600 | > 293 |
| I | 1.10 | 290 | < 45 | 268 | < 41 |
| II | 1.05 | 105 | < 45 | 100 | < 43 |
| III | 0.93 | 51 | < 45 | 52 | < 46 |

[a] Protein eluted from Q-Sepharose column between 0.12–0.25 M NaCl gradient. I: Sample obtained after first recycle through Q-Sepharose column equilibrated for protein non-adsorbing conditions (buffer: Tris Cl–urea containing 0.20 M NaCl, 19 mS cm$^{-1}$ conductivity). II and III: Samples obtained from the II and III stage of recycles. The endotoxin content in the initial sample was >360 (=0.125 × 2880) EU ml$^{-1}$, as sample solidified at 2880× dilution, whereas samples I, II and III did not solidify at lower dilution (360×), hence endotoxin content was <45 (=0.125 × 360) EU ml$^{-1}$.

### 3.5. Consistency of the final product

Immunoreactivity and the parameters of fluorescence spectra were used to determine the consistency of refolded CGβ(p) produced in different batches. The results of three batches of antigen are summarized in Table 3. The CGβ(p) produced in three batches reacted almost equally with the monoclonal antibody specific to nhCGβ. The intrinsic and extrinsic fluorescence spectral patterns were also consistent in all three batches of the antigen.

### 3.6. Immunogenicity

The antibody response to CGβ(p) produced in three different batches are shown in Fig. 4. In all cases maximum antibody titre obtained on the 9th week of immunization were 3.1 ± 0.80 μg ml$^{-1}$, 4.2 ± 0.70 μg ml$^{-1}$

Table 3
Immunoreactivity and parameters of fluorescence spectra[a]

| Batch no. | Immunoreactivity (absorbance at $OD_{492}$ in arbitary unit) | Intensity of emission spectra (arbitary unit) | | Blue shift of extrinsic spectra (nm) |
|---|---|---|---|---|
| | | Intrinsic | Extrinsic | |
| Sulfonated | 0.29 | 0.18 | 0.35 | – |
| I | 0.60 | 0.43 | 0.45 | 15 (535–520) |
| II | 0.75 | 0.45 | 0.49 | 12 (535–523) |
| III | 0.55 | 0.41 | 0.40 | 15 (535–520) |

[a] Results of three different batches of folding experiments are given. In each case, 120 μg ml$^{-1}$ sulfonated hCGβ was taken in TrisCl–urea buffer in presence of cysteamine–cystamine redox compounds at a molar ratio of 5:2.5. The folding was carried out at 4°C for 14 h, which was followed by slow removal of urea by dialysis over a period of 3 days. For immunoreactivity, 10 μg ml$^{-1}$ protein was quoted on ELISA plate, followed by reaction with primary and HRP-conjugated secondary antibodies, as described in Materials and Methods. The intrinsic fluorescence spectra was recorded in a 10 mm path-length cell in a total volume of 3 ml containing 2.98 ml of 20 mM phosphate buffer (pH 6.8), 5 mM KCl and 20 μl protein (500 nM). The extrinsic spectra was determined in the presence of 300 μM of 8-anilino-1-naphthalene-sulfonic acid (ANS). The samples were excited at 270 nm and 380 nm to record intrinsic and extrinsic spectra, respectively. Blue shifts of extrinsic spectra were calculated from the differences between emission spectra of sulfonated hCGβ (535 nm) to that in three batches of refolded protein.

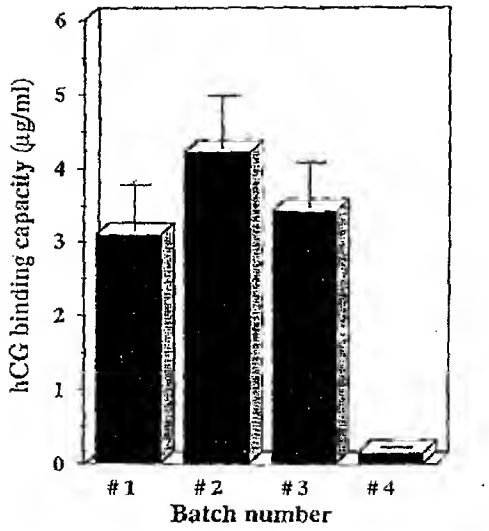


Fig. 4. The hCG-binding capacities of sera samples of Wistar rats ($n$ = 6). Wistar rats were immunized with 10 μg equivalent of alum adsorbed either CGβ(p), produced in three different batches, or alum adsorbed CGβ(up). Injections were given at 0, 4 and 8th weeks. Rats were bled on the 9th week of immunization, and anti-hCG antibody titres were assayed using hCG-dimer specific RIA. Batches no. 1–3: CGβ(p) produced in three different batches; batch no. 4: CGβ(up).

and 3.4 ± 0.60 μg $ml^{-1}$. The antibody titres subsequently dropped to 0.25–0.40 μg $ml^{-1}$ over a period of 21 weeks (data not shown). Interestingly, the injection of CGβ(up) elicited negligible (< 50 ng $ml^{-1}$) antibody response (Fig. 4, batch no. 4).

### 3.7. Lymphocyte proliferation

In order to evaluate potential T-cell response to native or recombinant antigen, CGβ(p)-primed mononuclear cells were cultured in the presence and absence of the antigen. The T-cell proliferation indices of four monkeys in response to the native or recombinant antigen are shown in Table 4. Out of four, three monkeys responded to both forms of the antigen. Incidentally, the monkey (MRA 474) which did not respond positively in the proliferation assay was a low responder one in terms of eliciting anti-hCG antibodies.

## 4. Discussion

The yield of β-subunit of human chorionic gonadotropin expressed in *E. coli* as inclusion bodies was superior to that obtained in other expression systems [7–10]. The recombinant antigen was purified to about 90% homogeneity in one-step anion exchange chromatography. The purified antigen was immunoreactive to native hCGβ-specific monoclonal antibody. The N-terminus sequence (20 aa) of the expressed subunit hormone was identical to that of the native protein, beginning with serine in position one. The amino acid sequence indicates that the expressed protein was adequately processed to remove N-terminus methionine. This was possible due to the presence of serine and lysine next to N-terminus methionine of the immature protein. It has been found that the in vivo N-terminus methionine is completely processed by methionine aminopeptidase, provided the side chain of the amino acid next to it is short and/or the amino acid is uncharged [23].

The hCGβ molecule contains 12 half-cystines which make six disulfide bonds. The thiol groups of proteins are unstable in vitro, as the disulfide bonds are often generated in the oxidizing environment at the time of processing. Spontaneous oxidation of the thiol groups results in random paired disulfide bonds with consequent possible changes in the conformation. In order to improve the yield of the monomeric form of the antigen, the thiol groups were first protected by reversible modification with sulfonate groups. The modified protein was refolded into almost 100% soluble monomeric CGβ(p) in presence of redox buffer. This was evident from the absence of oligomers in the non-reducing gel and from the elution profile of the gel filtration chromatogram. Previously it has been reported by other investigators that oligomers and large molecular aggregates were formed during in vitro folding of hCGβ [11]. It is important to mention here that these investigators did not protect thiol groups prior to folding. The formation of multimeric protein was

Table 4
Proliferation of CGβ(p) primed T-cells in response to nCGβ and CGβ(p)[a]

| Monkey | Proliferation index: Antigen nCGβ | Proliferation index: Antigen CGβ(p) | Antibody response |
|---|---|---|---|
| MRA 674 | 3.17 | 3.20 | +3 |
| MRA 551 | 2.95 | 3.63 | +2 |
| MRA 474 | 1.12 | 1.04 | 1 |
| MRA 550 | 2.86 | 2.56 | +2 |

[a] Each monkey was immunized with three injections each of 75 μg CGβ(p) adsorbed on alum. Peripheral blood was collected in 12th week of immunization, and presensitized mononuclear cells ($5 \times 10^5$ cells/well) were cultured in the presence of either nhCGβ or CGβ(p) at a concentration of 10 μg $ml^{-1}$. After 54 h, cells were pulsed with 1 μCi $^3$[H]-thymidine/well for 18 h, and its incorporation was determined. Proliferation index was calculated from the ratio of $^3$[H]-thymidine incorporation by the cells in presence and absence of antigen. Antibody response: +3 (anti-hCG titre of 1000–1500 ng $ml^{-1}$); antibody response: +2 (anti-hCG titre of 500–1000 ng $ml^{-1}$); antibody response: 1 (anti-hCG titre below 200 ng $ml^{-1}$).

believed to be due to inter-molecular disulfide bond formation and/or non-covalent association of the monomers. The monomeric CGβ(p) obtained in this investigation was a result of protection of the thiol groups, thiol/disulfide exchange in cysteamine–cystamine redox buffer in presence of 3 M urea, and stepwise removal of urea after refolding. The result also suggests that CGβ(p) is partially aggregated, if stored frozen.

Immunoreactivity of the refolded protein was changed with the redox status of the folding reaction buffer. The antigen–antibody reaction was maximum in the case of protein folded in 5 mM cysteamine and 2.5 mM cystamine redox buffer. Again, the magnitude of this reaction (optical density) was comparable to that with the native antigen. This demonstrates that a majority of the antigenic determinants were probably conserved in CGβ(p), if not completely.

All three batches of the antigen elicited significant and comparable amounts of antibodies in Wistar rats, indicating that CGβ(p) was consistent in immunogenic response. In an earlier paper [12] it was shown that the recombinant antigen elicited bioneutralizing antibodies comparable to that obtained using native antigen. The results of the immunogenicity test demonstrate that CGβ(p) had apparently the right conformation to present major B-cell epitopes on the surface. It has been reported earlier that B-cell epitopes in nhCGβ are, by and large, discontinuous, which depend primarily on the conformation of the molecule [24]. That CGβ(p) sensitized T-cells proliferated equally well in response to recombinant or native antigen also indicates that activated T-cells did recognize both forms of the antigen. This could be possible primarily due to conformational similarities between the recombinant and the native antigen.

In order to understand whether protection of the disulfide bonds followed by oxidative folding is essential for immunogenicity of the recombinant protein, the unprocessed antigen [CGβ(up)] was tested for antibody response under identical conditions. The anti-hCG antibody titre was significantly low as compared to that elicited by CGβ(p). This was probably due to improper folding of CGβ(up) leading to non-availability of B-cell epitopes on the surface. Both forms of the antigen [CGβ(p) and CGβ(up)] were then analysed for the structural variation, if any, by limited proteolysis and with the help of fluorescence spectra. The results showed sharp differences in the emission spectral patterns [12] between the two forms of the antigen indicating structural differences. This observation was supported by the finding that trypsin digested CGβ(p) and CGβ(up) liberate few dissimilar protein fragments on the SDS–PAGE gel (data not shown).

The present endeavour demonstrates refolding of inactive β-subunit of hCG to a functionally active antigen. The processed antigen [CGβ(p)] was highly immunogenic, and elicited bioneutralizing antibodies in experimental animals [12]. The recombinant antigen was produced in a highly purified form, and is expected to be cheaper than the available forms of the antigen. This report will bring impetus in the development of a cost-effective birth control vaccine.

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## References

[1] Talwar GP, Sharma NC, Dubey SK, Salahuddin M, Das C, Ramakrishna S, Kumar S, Hingorani V. Isoimmunization against human chorionic gonadotropin with conjugates of processed β-subunit of the hormone and tetanus toxoid. Proc Natl Acad Sci USA 1976;73:213–22.

[2] Thau RB, Sundaram K, Thorton YS, Seidman LS. Effects of immunization with β-subunit of ovine luteinizing hormone on corpus luteum functions in the rhesus monkey. Fert Steril 1979;31:200–4.

[3] Singh O, Rao LV, Gaur A, Sharma NC, Alam A, Talwar GP. Antibody response and characteristics of antibodies in women immunized with three contraceptive vaccines inducing antibodies against human chorionic gonadotropin. Fert Steril 1989;52:739–44.

[4] Jones WR, Judd SJ, Ing RMY, Griffin PD, Stevens VC. Phase-I clinical trials on a World Health Organization birth control vaccine. Lancet 1988;1:1295–8.

[5] Talwar GP, Singh O, Pal R, Chatterjee N, Sahai P, Dhall K, Kaur J, Das SK, Suri S, Buckshee K, Saraya L, Saxena BN. A vaccine that prevents pregnancy in women. Proc Natl Acad Sci USA 1994;91:8532–6.

[6] Stevens VC. Progress in the development of human chrionic gonadotropin antifertility vaccines. Am J Reprod Immunol 1996;35:148–55.

[7] Reddy VB, Becy AK, Garramore AJ, Velluci V, Lustbadar L, Bernstine EG. Expression of human chorionic gonadotropin in monkey cells using a single simian virus 40 vector. Proc Natl Acad Sci USA 1985;8:3644–8.

[8] Lustbader J, Birken S, Pollock S, Levinson L, Berstein E, Hoiung N, Canfield R. Characterization of the expression of products of recombinant human chorionic gonadotropin and subunits. J Bio Chem 1987;262:14204–12.

[9] Chen W, Shen QW, Bahl OP. Carbohydrate variant of the recombinant β-subunit of human chorionic gonadotropin expressed in baculovirus expression system. J Biol Chem 1991;266:4082–7.

[10] Srinivasan J, Singh O, Paul R, Lall L, Chakrobarty S, Talwar GP. In: Griffine PD, Johnson PM, editors. Local immunity in reproductive tract tissues. New Delhi: Oxford University Press, 1993. p. 477–81.
[11] Huth JR, Norton SE, Lockridge O, Shikone T, Hsueh AJW, Ruddon RW. Bacterial expression and in vitro folding of the β-subunit of human gonadotropin (hCGβ) and functional assembly of recombinant hCGβ with hCGα. Endocrinol 1994;135:911–8.
[12] Mukhopadhyay A, Bhatia PK, Majumdar SS. Preliminary studies with recombinant chorionic gonadotropin β-subunit produced in *Escherichia coli* for use as an antigen in a birth control vaccine. Am J Reprod Immunol 1998;39:172–82.
[13] Studier FW, Moffatt BA. J Mol Biol 1986;189:113–30.
[14] Panda A, Gorpade A, Mukhopadhyay A, Talwar GP, Garg L. High cell density fermentation of recombinant *Vibrio cholerae* for the production of B-subunit of *E. coli* enterotoxin. Biotechnol Bioeng 1995;45:245–50.
[15] Mukhopadhyay A. Inclusion bodies and purification of proteins in biologically active forms. In: Scheper T, editor. Adv Biochem Engg/Biotechnol, 56. Berlin: Springer, 1997. p. 61–110.
[16] Khandjian EW. Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membrane. Bio/Technol 1987;5:165–7.
[17] Maggio ET. Enzyme Immunoassay. Boca Raton, Fla.: CRC Press, 1981.
[18] Dighe RR, Moudgal NR. Use of α- and β-specific antibodies in studying interaction of hCG with Leydig cell receptors. Arch Biochem Biophys 1983;225:490–9.
[19] Shastri N, Dubey SK, Ragavan V, Salahuddin M, Talwar GP. Differential affinity of auto Pr-βhCG-TT antibodies for hCG and hLH. Contraception 1978;18:23–8.
[20] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[21] Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.
[22] Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Issaacs NW. Nature 1994;369:455–61.
[23] Ben-Bassat A. Method for removing N-terminal methionine from recombinant protein. In: Seethuram R, Sharma SK, editors. Purification and analysis of recombinant proteins. New York: Marcel Dekkar Inc, 1991. p. 147.
[24] Dirnhofer S, Madersbacher S, Bidart JM, Ten-kortenaar PB, Spottl G, Mann K, Wick G, Berger P. The molecular basis for epitopes on the free β-subunit of human chorionic gonadotropin, its carboxyl-terminal peptide and the hCGβ-core fragment. J Endocrinol 1994;141:153–62.